TOOLS AND METHODS USEFUL IN CHARACTERISING THE IMMUNOTOXIC ACTIVITY OF XENOBIOTIC SUBSTANCES

FIELD OF THE INVENTION

The invention relates to methods of in-vitro characterisation of a tested substance in order to ascertain its biological activity, especially in reference to its potential toxicity, and particularly immunotoxicity. The invention further relates to products used in the embodiment of such a test, and in particular a collection of cell lines and nucleic acids useful in their procurement.

BACKGROUND OF THE INVENTION

Every year there are new chemicals introduced into the occupational and environmental settings, which together with those already present may increase the risk of different adverse health effects. Epidemiological data clearly shows an increase in the prevalence of immunological disorders, which in part can be related to xenobiotic exposure as for example is in the case of allergic diseases, including asthma, among populations of industrialized countries. The immune system consists of very sensitive and specific network of cellular and humoral interactions that, when deregulated, causes the general malfunction of physiological processes of the host. Immunotoxicity is understood as the ability of a given compound to alter function of immune system of human or animal in a deleterious way.

Immunotoxicity testing is difficult and it is rather generally accepted that it cannot be accomplished with a single test. Strategies for immunotoxicity testing that have been developed and underwent the process of validation are based on a battery of tests. Frequently a "two tier" approach is proposed for immunotoxicity testing. In such a system the first tier consists of several screening tests detecting general abnormalities in the immune system such as morphological changes, while the second tier represents a more in depth evaluation of immune function following contact with given compound. Most of the tests for immunotoxicity that have been developed and validated employed experimental animals. These tests use a combination of *in vivo*, *ex vivo* and *in vitro* assays of immune functions and frequently involve isolated immune cells, usually lymphocytes. The difficulties related to predictive testing for immunotoxicity are in part related to multiple molecular and cellular targets of immunotoxin actions that have to be taken into account.

WO99/37142 concerns the production of transgenic animals for the study of Insulin-dependent Diabetes Mellitus. The document describes a method for producing a transgenic non-human mammal carrying a transgene encoding an immuno-inducible autofluorescent protein, said method comprising chromosomally incorporating a first DNA sequence encoding a cytokin promoter operatively connected to a second DNA sequence encoding said autofluorescent protein, such as Green Fluorescent Protein (GFP) or its enhanced variants (EGFP) into the genome of a non-human mammalian animal. A somatic cell from the transgenic mammal has been claimed inter alia.

WO02/22786 describes a cell line comprising a human cell line capable of producing a selected cytokine associated with an inflammatory response in humans, and transfected with a vector containing DNA encoding a cytokine regulatory factor (CRF) under the control of a promoter, and a vector containing DNA encoding a detectable-marker protein, under the control of a promoter responsive to cytokine induction. Disclosed cell line is useful for screening test compounds for anti-inflammatory activity, by its culturing under conditions in which CRF is overproduced in the transfected cells, the selected cytokine is induced, and the detectable-marker protein is produced at detectable levels, adding a test compound to the cultured cells, and observing any diminution in the level of the detectable-marker protein. Furthermore, an amount of dsRNA effective in stimulating cytokine production in the cytokine overproducing cells is added to the culture, and a priming agent such as phorbol myristate acetate (PMA), calcium ionophores, sodium butyrate, endotoxin, and cytokines is also added.

WO00/75660 provides methods of screening a test agent for the ability to reduce osteoclastic bone reabsorption. In a preferred embodiment, the methods involve screening the agent for the ability to inhibit tumor necrosis factor (TNF-alpha) expression through activity at an inhibitory TNF-alpha-responsive element (TNF-Re) in the tumor necrosis factor promoter or through activity at a complex formed by an estrogen receptor at TNF-Re. The document discloses screening a test agent for the ability to modulate osteoclastic bone reabsorption comprising: (a) contacting an estrogen receptor (ER) and a gene under the control of a tumor necrosis factor (TNF) modified promoter with a test agent; and (b) detecting a difference in the level of gene expression compared with a control cell. The method is useful for screening agents that reduce

osteoclastic bone reabsorption, and for the identification of compounds that modulate TNF-alpha expression resulting in reduced osteoclastic bone reabsorption.

WO0050872 provides systems, methods, screens, reagents and kits for an optical system analysis of cells to rapidly determine the distribution, environment, or activity of fluorescently labeled reporter molecules in cells for the purpose of screening large numbers of compounds for those that specifically affect particular biological functions.

WO0023615 discloses a method for extracting quantitative information relating to an influence on a cellular response in mechanically intact or permeabilized living cells, which method comprises recording variation in spatially distributed light emitted from a luminophore as a change in light intensity, measured by an instrument designed for the measurement of changes in fluorescence intensity. The luminophore, which is present in the cells, is capable of being redistributed in a manner which is related with the degree of the influence, and/or of being modulated by a component which is capable of being redistributed in a manner which is related to the degree of the influence.

There are attempts of *in vitro* immunotoxicity testing using isolated immune cells. In such tests immunosuppressive activity is expected when proliferative response or physiological functions like NK activity of immune cells are downregulated, and immunostimulation is expected when upregulation of these responses is detected. The possible immunomodulatory action is especially difficult to assess *in vitro* because of our limited understanding of molecular and cellular mechanisms mediating this effect. A specific problem for using *in vitro* tests lays in the fact that they generally do not take into account possible roles of metabolism in the xenobiotic's action.

Cytokines are generally defined as proteins secreted by cells that affect the behavior of other cells. They are produced by many cell types and through specific receptors affect activity of cells of different origin. It is believed that cytokines are critical regulators that orchestrate immune response by interconnecting dispersed elements of the immune system into one functional entity. They are grouped into families: the hematopoietins, the interferons, the chemokines, and the TNF family. T cells, among which one can distinguish function-related diversity, produce the greatest proportion of cytokines.

There is more and more evidence showing that at least certain types of immunotoxicity, such as those leading to hypersensitivity and autoimmunity are associated with modulation of the expression of particular cytokine genes in immune cells or non-immune cells. This effect seems to be important for immunotoxicity associated with heavy metals and amino acid derivatives linked to Eosinophilia-Myalgia Syndrome (USA in 1986). Derivatives of amino acids, which were putative ethiological agents in the epidemic mentioned above, were also shown to induce expression of a potent immunomodulatory cytokine, IL-5, in immune cells cultured *in vitro*. IL-4, the cytokine critical for development of allergic response, was secreted by lymphocytes and mast cells following contact with heavy metals, which represents a good correlation of xenobiotic action in vitro (upregulation of IL-4 in immune cells) and *in vivo* (upregulation of IgE in experimental animals). Certain cytokines expressed by non-immune cells are also important signals modulating immune response. For example, some chemical allergens were reported to stimulate keratinocytes to express particular cytokines such as IP-10, MIP-2, IL-1β, and IL-10. Some types of immunosuppression can also involve the modulation of cytokine expression like that observed with azathioprine or cyclosporin A, which inhibit IL-2 expression in lymphocytes.

In light of the presented current state of technology, it is desirable to develop new methods of (immuno)toxicity testing in vitro, which would not only indicate perturbations of the immune system but also allow the elucidation of the potential mechanism of immunomodulation. This approach would utilize the knowledge about pleiotropic activities of cytokines that regulate different processes of immune system.

Thus, the main objective of this invention is a development of a new system of characterising the biological activities of xenobiotics in vitro. A particular goal of the invention is to facilitate easy and reliable tests for their toxicity, particularly immunotoxicity.

SUMMARY OF THE INVENTION

The present invention relates to an isolated nucleic acid molecule coding an expression box with the formula S_1-S_2-S_3 where: S_1 is a promoter sequence, or it is absent, S_2 is a known reporter gene sequence, S_3 is a regulatory 3'UTR sequence, or it is absent, where the promoter sequence and the regulatory 3'UTR sequence originate from a known cytokine gene, and are

the controlling sequences of said cytokine. In a preferential embodiment of the present invention, the reporter gene is a gene coding a Green Fluorescent Protein, preferentially selected from its variants: d1EGFP, d2EGFP, EGFP or EGFP-F. In a preferential embodiment of the present invention, the promoter sequence and regulatory 3'UTR sequence originate from a cytokine selected from among the following: IL-1β, IL-2, TNFα, IL-4, IL10 or INFγ. In particular, the nucleic acid molecule can be an expression box contained in a plasmid selected from among the following: p1-5'IL1β/d1EGFP-N1 (SEQ ID NO:1), p2-5'IL1β/d1EGFP-N1 (SEQ ID NO:2), p3-5' IL1β/d1EGFP-N1 (SEQ ID NO:3), p4-5'IL1β/d1EGFP-N1 (SEQ ID NO:4), p1-5'3' IL1β/d1EGFP-N1 (SEQ ID NO:5), p2-5'3'IL1β/d1EGFP-N1 (SEQ ID NO:6), p3-5'3'IL1β/d1EGFP-N1 (SEQ ID NO:7), p4-5'3'IL1β/d1EGFP-N1 (SEQ ID NO:8), p1-5'IL2/EGFP-1 (SEQ ID NO:9), p1-5'IL2/d2EGFP-1 (SEQ ID NO:10), p1-5'3'IL2/d2EGFP-1 (SEQ ID NO:11), p1-3'TNFα/d1EGFP-N1 (SEQ ID NO:12), p2-3'TNFα/EGFP-F (SEQ ID NO:13), p3-3'TNFα/EGFP-F (SEQ ID NO:14), p1-5'TNFα/d1EGFP-N1 (SEQ ID NO:15), p1-5'3'TNFα/d1EGFP-N1 (SEQ ID NO:16), p1-3'IL4/d1EGFP-N1 (SEQ ID NO:17), p2-3'IL4/EGFP-F (SEQ ID NO:18), p3-3'IL4/EGFP-F (SEQ ID NO:19), p4-3'IL4/CA-EGFP (SEQ ID NO:20), p5-3'IL4/d1EGFP-N1 (SEQ ID NO:21), p1-5'IL4/EGFP-1 (SEQ ID NO:22), p1-5'IL4/d1EGFP-N1 (SEQ ID NO:23), p2-5'IL4/EGFP-1 (SEQ ID NO:24), p2-5'IL4/d1EGFP-N1 (SEQ ID NO:25), p1-5'3'IL4/EGFP-1 (SEQ ID NO:26), p1-5'3'IL4/d1EGFP-N1 (SEQ ID NO:27), p2-5'3'IL4/d1EGFP-N1 (SEQ ID NO:28), p1-5'INFγ/EGFP-1 (SEQ ID NO:29), p1-5'INFγ/d2EGFP-1 (SEQ ID NO:30), p1-5'3'INFγ/d2EGFP-1 (SEQ ID NO:31), p1-5'IL10/EGFP-1 (SEQ ID NO:32), p1-5'3'IL10/EGFP-1 (SEQ ID NO:33), p2-5'IL10/d2EGFP-1 (SEQ ID NO:34), p2-5'3'IL10/d2EGFP-1 (SEQ ID NO:35).

Another aspect of the present invention also relates to an expression vector, containing a nucleic acid molecule coding an expression box according to the present invention, as defined above. In a particular embodiment, the expression vector is a plasmid selected from among the following: p1-5'IL1β/d1EGFP-N1 (SEQ ID NO:1), p2-5'IL1β/d1EGFP-N1 (SEQ ID NO:2), p3-5' IL1β/d1EGFP-N1 (SEQ ID NO:3), p4-5'IL1β/d1EGFP-N1 (SEQ ID NO:4), p1-5'3' IL1β/d1EGFP-N1 (SEQ ID NO:5), p2-5'3'IL1β/d1EGFP-N1 (SEQ ID NO:6), p3-

5'3'IL1β/d1EGFP-N1 (SEQ ID NO:7), p4-5'3'IL1β/d1EGFP-N1 (SEQ ID NO:8), p1-5'IL2/EGFP-1 (SEQ ID NO:9), p1-5'IL2/d2EGFP-1 (SEQ ID NO:10), p1-5'3'IL2/d2EGFP-1 (SEQ ID NO:11), p1-3'TNFα/d1EGFP-N1 (SEQ ID NO:12), p2-3'TNFα/EGFP-F (SEQ ID NO:13), p3-3'TNFα/EGFP-F (SEQ ID NO:14), p1-5'TNFα/d1EGFP-N1 (SEQ ID NO:15), p1-5'3'TNFα/d1EGFP-N1 (SEQ ID NO:16), p1-3'IL4/d1EGFP-N1 (SEQ ID NO:17), p2-3'IL4/EGFP-F (SEQ ID NO:18), p3-3'IL4/EGFP-F (SEQ ID NO:19), p4-3'IL4/CA-EGFP (SEQ ID NO:20), p5-3'IL4/d1EGFP-N1 (SEQ ID NO:21), p1-5'IL4/EGFP-1 (SEQ ID NO:22), p1-5'IL4/d1EGFP-N1 (SEQ ID NO:23), p2-5'IL4/EGFP-1 (SEQ ID NO:24), p2-5'IL4/d1EGFP-N1 (SEQ ID NO:25), p1-5'3'IL4/EGFP-1 (SEQ ID NO:26), p1-5'3'IL4/d1EGFP-N1 (SEQ ID NO:27), p2-5'3'IL4/d1EGFP-N1 (SEQ ID NO:28), p1-5'INFγ/EGFP-1 (SEQ ID NO:29), p1-5'INFγ/d2EGFP-1 (SEQ ID NO:30), p1-5'3'INFγ/d2EGFP-1 (SEQ ID NO:31), p1-5'IL10/EGFP-1 (SEQ ID NO:32), p1-5'3'IL10/EGFP-1 (SEQ ID NO:33), p2-5'IL10/d2EGFP-1 (SEQ ID NO:34), p2-5'3'IL10/d2EGFP-1 (SEQ ID NO:35).

Another aspect of the present invention also relates to a single-celled host transformed or transfected with a DNA molecule according to the present invention, as defined above. The initial cells used to obtain the single-celled host can be selected from among the group encompassing bacteria, yeast, mammalian cells, plant cells, insect cells, as well as eukaryotic cell lines. In a particular embodiment it is an immortal mammalian cell line, preferentially descendant from cells of the immune system, or for example it is a cell line selected from among T cell leukemia cells, thymoma, mast cells, macrophage-monocytes, fibroblasts and keratinocytes; for example, a cell line selected from among: EL4, BW5147.3, C57.1, J774A.1, 3T3 L1, MC/9 and HEL-30. In an embodiment, as a result of recombination, the natural cytokine gene extant in the host cell has been replaced by the DNA molecule according to the present invention, as defined above. In a particular embodiment, the single celled host is a cell line selected from among C/p1-5'3'TNFα-dEGFP/2 (deposited in ECACC, Accession No. 3091202), EL/p1-5'IL2-dEGFP/6 (deposited in ECACC, Accession No. 3091204), EL/p2-5'IL4-dEGFP/2 (deposited in ECACC, Accession No. 3091205), EL/p1-5'IFNγ-dEGFP/3 (deposited in ECACC, Accession No. 3091206), EL/p2-5'IL10-

dEGFP/5 (deposited in ECACC, Accession No. 3091207), J/p4-5'IL1β-dEGFP/4 (deposited in ECACC, Accession No. 3091208).

Another aspect of the present invention also relates to a collection of cell lines recognizable in that it contains at least one cell line according to the present invention, as mentioned above, as well as at least one positive control cell line showing a constitutive expression of the reporter gene sequence. The positive control cell line originates from cells selected from the group encompassing bacteria, yeast, mammalian cells, plant cells, insect cells, as well as eukaryotic cell lines. In another embodiment, the positive control cell line is an immortal mammalian cell line. Preferentially, in the positive control cell line the reporter gene sequence is operationally bound to the regulatory sequence giving constitutive expression, where preferentially it contains at least one element from among the following: 3'UTR GAPDH, promoter/enhancer CMV, promoter-actin or derivatives thereof. In a particular embodiment, the positive control cell line is transformed or transfected with a plasmid selected from among the following: p1-3'GAPHD/d1EGFP-N1 (SEQ ID NO:36), p2-3'GAPHD/EGFP-F (SEQ ID NO:37), p3-3'GAPDH/EGFP-F (SEQ ID NO:38), pCA-EGFP-F (SEQ ID NO:39), pCA-d1EGFP (SEQ ID NO:40). In the example embodiment, the positive control cell line is the C/pCA-EGFP-F/2 line (deposited in ECACC, Accession No. 3091201) or EL/pCA-dEGFP/9 (deposited in ECACC, Accession No. 3091203). In a particularly preferential embodiment the collection of cell lines is a cell-chip.

Another aspect of the present invention also relates to a method of obtaining the characteristics of the tested substance characterised in that

a) the tested substance is put into contact with the cell line according to the present invention, or a cell line belonging to a collection of cell lines according to the present invention, as defined above,

b) it determines a change in the level of expression of a reporter gene caused by the tested substance,

c) the change in the level of expression described in (b) is accepted as characteristic of the tested substance.

In particular, GFP or one of its known variants is used as a reporter gene, and in stage (b) changes in the intensity of fluorescence are measured. In a particular embodiment of the method in stage (b), changes in th level of expression of the reporter gene is studied for each cell line in the collection. In stage (c) an expression profile characterizing the tested substance is obtained, based on results obtained from cell lines belonging to the collection. In a preferential embodiment of the method, stage (a) and/or (b) are performed simultaneously on all cells belonging to the collection. Stages (a) and (b) may be performed in an automated manner, and in stage (c), the results obtained may be computer analysed. In one of the possible embodiments the results obtained in stage (c) from the tested substance are compared to results obtained from substances of known properties. In a particular embodiment, stage (a) is performed in the presence of an expression modulator. The modulator of expression is an activator inducing the expression of the reporter gene, for example selected from among the following: PMA, ionomycin, calcium ionophore, LPS or a combination thereof. In a particular embodiment of the method, the characteristics obtained in stage (c) are used to ascertain the biological activity of the tested substance. In particular, in stage (c), the results of expression level measurements obtained from the tested substance are compared to results obtained from a reference substance of known biological activity. In a particular embodiment of this aspect, the studied biological activity is toxicity, particularly immunotoxicity.

Another aspect of the present invention relates to the use of a cell line according to the present invention, or a collection of cell lines according to the present invention, as defined above, to study the biological activity of the tested substance. In a particular embodiment of this aspect of the present invention, the studied biological activity is toxicity, particularly immunotoxicity.

Another aspect of the present invention relates to the use a cell line according to the present invention or a collection of cell lines according to the present invention, as defined above, to obtain the characteristics of the tested substance.

The presented system does not involve experimental animals, but instead is based on a number of immortalized cell lines representing different phenotypes of cells which regulate

immune response in vivo. These cell lines have been tested in a uniform high throughput system for the expression of a number of cytokine genes. For this purpose, specialized reporter cell lines have been generated and used to detect signals, which upregulate and downregulate the expression of immunomodulatory cytokines upon contact of these cells with tested compounds e.g. a xenobiotic. Reporter cell lines have been prepared by genetic modification of cell lines in vitro. Each obtained cellular clone has been characterized and tested using a set of model immunotoxins, which have demonstrated adverse effects, in vivo. The entire panel of reporter cell lines was then pre-validated as a tool for testing immunotoxicity using data derived from already established tests as a reference.

The main achievement of this invention is the construction of a new tool for the detection of possible (immuno)toxicity associated with xenobiotics by performing an in vitro test. This tool consists of a series of reporter cell lines that regulate the expression of a transgene coding for fluorescent protein in the same way as they regulate the expression of cytokines. Expression of a fluorescent protein allows for the fast (near real time) detection of intracellular signals leading to changes in cytokine gene expression upon contact of the cells with the tested substance.

In a preferential embodiment of the invention, a single assay employing a cell chip allows the detection of possible interference of the tested xenobiotic with different tissue-specific molecular targets, such as signal transduction molecules and transcription factors, and to generate a compound specific pattern of response.

A pre-validation of a new testing system against data based on existing tests was performed. In particular this includes the standardization of the cell chip against several "model xenobiotics" (substances already known for their immunotoxic activities observed in vivo). This information suggests the conclusion that development of specialized genetically modified cell lines provides a useful biological marker for immunotoxicity testing and this technology might be expanded into other area of alternative toxicity testing.

The present method may also find an application as a facile method of characterising chemical substances, for example at the stage of screening a library of new chemical compounds, or in the search for new drugs.

DETAILED DESCRIPTION OF THE FIGURES

Figures 1-19 show schematics of plasmids obtained according to the procedure described in Example 1. The sequences of these plasmids have been disclosed in the Sequence Listing.

Figure 20 and 21 shows results of cell vialbility testing mentioned in Example 7.

Figure 22-25 present the results of preliminary tests of reporter cell lines,which has been described in Example 7.

Figure 26-32 present results of testing the effect of certain substances using the prototype cell chip.

DETAILED DESCRIPTION OF THE INVENTION

In order to obtain reporter cell lines of the desirable phenotype, immortalized mammalian cell lines derived from different lineages of the immune system, as well as some non-immune cell types were tranfected with reporter genes encoding fluorescent protein. Reporter genes consist of sequences coding for fluorescent protein and regulatory sequences controlling cytokine expression. Different experimental approaches were used to generate reporter cell lines. Example 1 and Example 2 describe approaches employing a stable transfection system in which reporter genes are randomly incorporated into chromosome. Possible shortcomings detected in reporter cell lines obtained employing this experimental protocol will be corrected later by modifications of the original expression vectors and generation another series of transfectants in Example 3 and Example 4. In order to reproduce the complex regulatory mechanisms controlling cytokine gene expression in a given lineage of immune cells gene targeting technologies were employed next in Example 5 and Example 6. Reporter cell lines obtained with these genetic modifications were characterized for phenotype and were tested using selected xenobiotics in Example 7. Next, the set of selected cell lines, the fluorescent cell chip, was assembled and tested. This cell chip underwent the process of testing and pre-validation described in Example 8. Patterns of signals detecting the modulation of cytokine expression obtained with a selected xenobiotic in the fluorescent cell chip were then compared to results obtained with existing tests, and with available clinical data.

The main aspect of the invention involves a preparation of the system for *in vitro* immunotoxicity testing. This requires preparation of cell lines, in which signals regulating

expression of several cytokines will generate increase in specific fluorescence. The following combinations of cytokine genes and cell lines presented in Table I were used as a starting point for the development of a "cell chip".

Table I

Cytokine gene	Cell lines transfected with a reporter plasmid	Reporter gene
IL-2	T cell leukemia (EL4)	EGFP/dEGFP
	Thymoma (BW5147.3)	EGFP/dEGFP
IFN-γ	T cell leukemia (EL4)	EGFP/dEGFP
	Thymoma (BW5147.3)	EGFP/dEGFP
IL-4	T cell leukemia (EL4)	EGFP/dEGFP
	Mast cells (C57.1)	EGFP/dEGFP
TNF-α	Macrophage-monocytes (J774A.1)	EGFP/dEGFP
	Fibroblasts (3T3 L1)	EGFP/dEGFP
	Mast cells (MC/9, C57.1)	dEGFP
IL-1β	Macrophage-monocytes (J774A.1)	dEGFP
	Keratinocytes (HEL-30)	dEGFP
	Fibroblasts (3T3 L1)	dEGFP
Control (CMV promoter o	Mast cells (C57.1)	dEGFP
	Fibroblasts (3T3 L1)	EGFP/dEGFP
β-actin promoter)	Keratinocytes (HEL-30)	dEGFP
	T cell leukemia (EL4)	EGFP/dEGFP
	Thymoma (BW5147.3)	EGFP
	Macrophage-monocytes (J774A.1)	EGFP/dEGFP

For testing and pre-validation of the prototype cell chip, compounds from the list of chemicals with already established immunotoxic potential, and in some cases with a partially understood mechanism of action, were employed. The list of compounds is presented in Table II.

Table II

Substance	Type of activity	Possible action on cytokine gene expression
Cyclosporin	Suppression	Inhibition of cytokine production
Dioxin	Suppression	Alteration of cytokine production (inhibition and/or activation)
Pentamidine	Suppression	Inhibition of cytokine expression
Rapamycin	Suppression	Alterations in transcriptional mechanisms regulating cytokine production
Thalidomide	Suppression	Alterations in transcriptional mechanisms regulating cytokine production
TBTO, Bis-(tri-n-butyltin)-oxide	Suppression	Possible inhibition of cytokine production
House dust mite allergen	Antigen	Unknown
Benzocaine	Allergen	Unknown
Penicilline	Allergen	Unknown
TDI, Toluene-2,4-diisocyanate	Allergen	Induction of TH2 cytokine expression
MDI, Diphenylmethane-4,4'-diisocyanate	Allergen	Unknown
DNCB, Dinitrochlorobenzene	Contact allergen	Induction of cytokine expression
Dicyclohexyl methane-4,4'-diisocyanate	Contact Allergen	Unknown
HCB, Hexachlorobenzen	Immunostimulation Induction of autoimmunity Immunomodulation	Activation of cytokine expression
HgCl2	Induction of autoimmunity Immunomodulation	Induction of TH2 cytokine expression
Platinum salt (Tetrachloroplatinate)	Immunostimulation Respiratory allergen	Unknown
SDS	Irritant	Unknown

According to the one of the embodiments of the present invention, unique cell lines were obtained and selected, which may serve to embody the method according to the present invention as well as being an example collection of cell lines according to the present invention. Said lines were deposited in the European Collection of Cell Cultures (ECACC). They are presented in Table III.

Table III

Name	Provisional Accession No assigned by ECACC	Parental cell line	Transfected with
C/pCA-EGFP-F/2	3091201	C57.1	pCA-EGFP-F
C/p1-5'3'TNFα-dEGFP/2	3091202	C57.1	p1-5'3'TNFα/d1EGFP-N1
EL/pCA-dEGFP/9	3091203	EL4	pCA-d1EGFP
EL/p1-5'IL2-dEGFP/6	3091204	EL4	p1-5'IL2/d2EGFP-1
EL/p2-5'IL4-dEGFP/2	3091205	EL4	p2-5'IL4/d1EGFP-N1
EL/p1-5'IFNγ-dEGFP/3	3091206	EL4	p1-5'IFNγ/d2EGFP-1
EL/p2-5'IL10-dEGFP/5	3091207	EL4	p2-5'IL10/d2EGFP-1
J/p4-5'IL1β-dEGFP/4	3091208	J774A.1	p4-5'IL1β/d1EGFP-N1

each of these lines has been selected from among the collection of cell lines obtained on the basis of favourable characteristics summarised below. These were determined in accordance with the detailed description included in each example.

C/pCA-EGFP-F/2 is a control cell line. It was tested for EGFP fluorescence by FACS and fluorescence microscopy. It shows stable expression of EGFP.

EL/pCA-dEGFP/9 is a control cell line. It was tested for EGFP fluorescence by FACS and fluorescence microscopy. It shows stable expression of EGFP

C/p1-5'3'TNFα-dEGFP/2 is a cell line in which EGFP expression parallels the expression of TNF-. EGFP fluorescence is stimulated with PMA/ionomycine and antigen/IgE. This cell line demonstrated weak inhibition of EGFP fluorescence in the presence of immunossuppresive substances. Thus it detects the inhibitory action of chemical compounds, which could result in immunosuppression.

EL/p1-5'IL2-dEGFP/6 is a cell line in which EGFP expression parallels expression of IL-2. EGFP fluorescence is stimulated with PMA/ionomycine. This cell line demonstrated inhibition of EGFP fluorescence in the presence of immunossuppresive substances. Thus it detects the inhibitory action of chemical compounds, which could result in immunosuppression.

EL/p1-5'IFNγ-dEGFP/3 is a cell line in which EGFP expression parallel expression of IFN γ. EGFP fluorescence is stimulated with PMA/ionomycine. This cell line demonstrated inhibition of EGFP fluorescence in the presence of immunossuppresive substances. Thus it detects the inhibitory action of chemical compounds, which could result in immunosuppression. This cell line demonstrated increased EGFP fluorescence in the presence of a possible immunomodulator. Thus it detects the activatory action of chemical compounds, which could result in immunomodulation.

EL/p2-5'IL4-dEGFP/2 is a cell line in which EGFP expression parallels expression of IL-4. EGFP fluorescence is stimulated with PMA/ionomycine

EL/p2-5'IL10-dEGFP/5 is a cell line in which EGFP expression parallels expression of IL-10. EGFP fluorescence is stimulated with PMA/ionomycine.

J/p4-5'IL1β-dEGFP/4 is a cell line in which EGFP expression parallels expression of IL-1. EGFP fluorescence is stimulated with LPS. This cell line demonstrated increase in EGFP fluorescence in the presence of immunostimulatory substance. Thus it detects the action of a chemical compound, which could result in immunomodulation.

In order to present the sense of invention, the description of invention is expanded by examples 1-8. However, it is not our intention to introduce claims limited to embodiments described in examples, because basing on presented sense of invention combined with knowledge generally available, experts will be able to prepare other variants comprised in defined claims.

Example 1. Construction of expression vectors for stable transfection

To generate DNA constructs in which the expression of a reporter fluorescent protein depends on regulatory sequences derived from different cytokine genes. These expression vectors are necessary tools for the genetic modification of cell lines and will be used in Example 2.

Methodology and study materials

Several sequences containing regulatory elements of promoter regions from 5' upstream of cytokine genes, including IL-2, IFN-γ, IL-4, IL-1α, and TNF-α were collected. DNA was acquired from three sources; clones that are available in the public domain, PCR amplification of desired DNA fragments from genomic DNA followed by PCR product cloning, and

chemical synthesis of oligonucleotides. Commercially available plasmids containing the GFP gene were used as the backbone of the construct. The promoter sequence of interest was cloned immediately upstream of the GFP transcription start site, using standard techniques of directional cloning with synthetic oligonucleotide adapters when necessary. Positive clones were selected and the DNA sequences of plasmids were verified with automated DNA sequencing.

Next, the regulatory sequences in 3'UTR fragments of the same selected cytokine genes were collected. The plasmids from the first series of GFP constructs were used to develop the second series of constructs. The 3'UTR sequences covering the polyA signal and mRNA stabilizing signals present in the original GFP plasmid were replaced with DNA coding for 3' UTR sequences of cytokine gene inserted immediately downstream of GFP stop codon. Synthetic oligonucleotide adapters were used when necessary. Positive clones were selected and the DNA sequences of plasmids were verified by automated DNA sequencing.

Consequently, construction of at least 5 expression vectors containing only promoter sequences and at least 5 expression vectors containing both promoter and downstream (3'UTR) sequences derived from cytokine genes were obtained.

Collection of 5' upstream regulatory sequences

Sequences containing regulatory elements of the promoter region from 5' upstream of **IL-4** cloned into plasmid pCAT were obtained as a gift from Dr. Melissa Brown (Atlanta Univeristy, Atlanta, USA). Promoter sequences for: IL-2, IFN-γ, IL-1β, and TNF-α were obtained by PCR based cloning using mouse genomic DNA isolated from Balb/c as a template.

For cloning of **IL-2** and **IFN-γ** derived sequences, genomic DNA was prepared from the tail tip of a Balb/c mouse. This DNA served as a template for the PCR amplification. PCR primers were designed based on the genomic sequences of murine IL-2 (X52618) and IFN-γ (M28381), available from GenBank. The primers were designed to encompass the upstream region of IL-2 from position –2686 to +25, and IFN-γ from the position –2001 to +34, relative to the transcription start site denoted as +1. The rationale was to include a large upstream region in order to include many putative regulatory elements. The primers (TAG Copenhagen)

were then used for PCR amplification using the "PCR core kit" (Roche Biochemicals). PCR products of the correct size were excised from an agarose gel, purified using a QiaQuick gel extraction kit (Qiagen), checked for integrity on an agarose gel and ligated into the PCR cloning vector pGEM-T Easy (Promega). The ligation was transformed into JM109 *E.coli* cells and colonies containing inserts were selected using ampicillin. Plasmid DNA was extracted from a number of clones using a QiaSpin miniprep kit (Qiagen). The plasmids were then screened for the presence and orientation of the PCR product using multiple restriction digests. Finally, the presence and orientation of the IL-2 and IFN-γ 5' upstream regulatory regions were confirmed by automated sequencing (ABI 377 DNA sequenator) from both ends using primers annealing to the vector.

It was decided to clone promoter of IL-1β instead of IL-1α. The reason to change the original plan was the fact that unlike the sequence of the IL-1α promoter, the entire DNA sequence of the murine IL-1β promoter was available in genetic databases. Although there might be differences in transcriptional regulation of *IL-1 alpha* and *IL-1 beta* genes IL-1α and IL-1β act trough the same cell surface receptor, and have similar functions.

For cloning of **TNF-α** and **IL-1β** derivative sequences, genomic DNA was prepared from the liver of a Balb/c mouse. This DNA served as a template for PCR amplification. PCR primers were designed based on the genomic sequence of murine TNF-α (U066950) and IL-1β (X04964), available in databases. A 511 bp fragment of mouse IL-1β promoter (-500/+11) was amplified by PCR using Taq polymerase (MBI Fermentas). The PCR product was cloned into TA-cloning vector pTAdvance (Clontech). A 4104 bp of fragment of IL1-β promoter (-4093/+11) was amplified by PCR using the high-fidelity AccuTaq LA thermostable polymerase (Sigma). The PCR product was ligated into the pCR-Blunt II-TOPO blunt-end cloning vector (Invitrogen). The ligation mixture was transformed into JM109 *E.coli* cells and colonies containing inserts were selected using kanamycin. Bacterial clones were screened for the presence of IL1-β promoter using PCR. Plasmid DNA was extracted from a number of bacterial clones using Plasmid Miniprep Plus kit (A&A Biotechnology) and the presence and orientation of the IL1-β promoter containing insert was verified using multiple restriction digests. Finally, the sequence and orientation of the IL-1β 5' upstream regulatory regions were

confirmed by automated sequencing (ABI 377 DNA sequenator) using primers annealing to the vector. A 2276 bp fragment of the mouse TNF-α promoter (-2013/+263) was obtained using similar experimental protocol. Briefly, PCR was performed using AccuTaq LA polymerase and the PCR product was cloned into pCR-Blunt II-TOPO vector. Following bacterial transformation the bacterial clones were screened for the presence and orientation of the TNF-α promoter region using PCR and restriction analysis. Finally, the sequence and orientation of the TNF-α promoter was confirmed by automated sequencing.

Generation of the first series of reporter constructs

The first series of reporter constructs consists of a number of plasmids where expression of the reporter gene is driven by promoter sequences of cytokines: IL-4, IL-2, IFN-γ, IL-1β, and TNF-α. All reporter constructs are based either on the plasmid pEGFP-1 (Clontech) containing enhanced green fluorescent protein (EGFP) as a reporter gene or on the plasmid pd1EGFP-N1 (Clontech) containing destabilized enhanced green fluorescent protein (d1EGFP) as a reporter gene.

To obtain the GFP reporter construct under the control of the **IL-4** promoter a 120 bp DNA fragment containing the minimal IL-4 promoter sequence was excised from –87IL-4 pCAT plasmid using HindIII and XbaI restriction enzymes. This DNA was then ligated into pTAdvance plasmid (Clontech). In the next step, the IL-4 promoter was excised from pTAdvance plasmid using HindIII and ApaI, purified by agarose electrophoresis and ligated into the pEGFP-1 plasmid digested with the same enzymes. Plasmid DNA was isolated from kanamycin resistant bacterial clones and the integrity of the reporter construct was confirmed by ApaI / HindIII and HincII / HindIII digestions, and by sequencing. Obtained plasmid was named **p1-5'IL4/EGFP-1.**

To obtain the GFP reporter constructs under the control of **IL-2** and **IFN-γ** promoters the PCR cloned IL-2 and IFN-γ 5' regulatory regions were excised from pGEM-T Easy plasmids containing the inserts in negative orientation, using the restriction enzymes ApaI and PstI. The obtained DNA fragments were purified by agarose gel electrophoresis and a QiaQuick gel extraction kit (Qiagen), checked for the integrity on an agarose gel and ligated into the vector pEGFP-1 digested with ApaI and PstI in the multiple cloning site immediately

upstream of the gene encoding EGFP. Following transformation, colonies were picked and isolated plasmids were screened for insertion of the IL-2 or IFN-γ promoters using multiple restriction digests. The integrity of the reporter constructs was confirmed by automated sequencing (ABI 377 DNA sequenator) from both ends using primers annealing to the vector. The obtained plasmids were named **p1-5'IL2/EGFP-1** and **p1-5'INFγ/EGFP-1**.

To obtain the GFP reporter construct under the control of the **IL-1β** promoter, a 511 bp fragment of mouse IL-1β 5' regulatory region excised from pTAdvance plasmid using AseI and EcoRV (NEB) was used to replace the CMV promoter in the pd1EGFP-N1. The CMV promoter was removed using AseI and Eco47III restriction enzymes (NEB, MBI Fermentas). The presence of the IL-1β 5' regulatory region was confirmed by PCR and BamHI (NEB) digest. The integrity of the reporter construct, named **p1-5'ILβ/d1EGFP-N1,** was confirmed by automated sequencing using primers annealing to the vector. Next, a 4104 bp fragment of IL1-β 5' regulatory region was excised from pCR-Blunt II-TOPO vector using EcoRV and KpnI (NEB). The pd1EGFP-N1 vector was modified by removal of the CMV promoter using AseI and NheI (NEB) restriction, treated with Mung Bean Nuclease (NEB) to generate blunt ends, and ligated. EcoRV/KpnI IL1-β promoter was then ligated with modified pd1EGFP-N1 plasmid restricted with SmaI and KpnI. The presence of IL-1β promoter was confirmed by PCR and restriction with Eco88I (MBI Fermentas). The integrity of the obtained plasmid, named **p3-5'IL1β/d1EGFP-N1,** was confirmed by sequencing.

To obtain the GFP reporter construct under the control of the **TNF-α** promoter, a 2526 bp TNF-α 5' regulatory region was excised from pCR-Blunt II-TOPO with the use of AseI/EcoRV (NEB). CMV promoter in pd1EGFP-N1 plasmid was excised by Ecl136II/VspI (MBI Fermentas) digestion. Both the 2526 bp insert and the resulting 4251 bp promoterless vector were purified by agarose gel electrophoresis and ligated using T4 ligase (Gibco BRL). Following screening of the bacterial clones for a proper ligation product a 6777 bp plasmid, named **p1-5'TNFα/d1EGFP-N1**, was identified and purified. The sequence was verified using automated sequencing. This plasmid contains the entire TNF-α promoter region followed by 107 bp of TNF-α ORF and 80 bp of randomly generated linker at the beginning of d1EGFP ORF.

To obtain the EGFP reporter construct under the control of the actin promoter, a CAG promoter containing CMV enhancer and a chicken beta-actin promoter was excised with AseI and Eco47III restriction enzymes from commercially available plasmid pQE-TriSystem (Qiagen) and ligated into pEGFP-F plasmid (Clontech) in the place of CMV promoter (cut out with AseI and Eco47III restriction enzymes), upstream of EGFP coding sequence. The sequence of the resultant construct, **pCA-EGFP-F,** was confirmed by restriction enzyme maping.

Collection of 3' downstream regulatory sequences

For cloning of 3'UTR regulatory sequences derived from IL-4, IL-2, IFN-γ, IL-1β, and TNF-α the respective sequences were amplified by PCR from genomic DNA obtained from the tail tips (IL-2 and IFN-γ) or from the livers (IL-4, IL-1β, and TNF-α) of Balb/c mice.

To clone **IL-4 3'UTR,** primers based on the IL-4 genomic sequence (X05253) were designed to encompass the entire IL-4 3'UTR, including the polyadenylation signal. Using these primers, a 154 bp long DNA fragment was amplified by PCR and cloned into pTAdvance vector (Clontech). The sequence of the cloned IL-4 3'UTR was verified by automated sequencing.

To obtain the regulatory elements present in the **3'UTR** of **IL-2** and **IFN-γ**, the PCR primers were designed to encompass the 3'UTRs, including the translational stop codon and the polyadenylation signal. Primers were designed based on the mRNA sequences of IL-2 (X01772) and IFN-γ (K00083), available in databases. The downstream primers were designed to include AflII restriction sites. The 357 bp IL-2 3'UTR and the 645 bp IFN-γ 3'UTR amplified by PCR from genomic DNA were cloned non-directionally into the pGEM-T Easy vector (Promega). The identity and orientation of the inserts were then confirmed using multiple restriction digests.

To clone **IL-1β 3'UTR**, primers based on IL-1βgenomic sequence (X04964) were designed to encompass the entire IL-1β3'UTR, including the polyadenylation signal. Using these primers, a 455 bp long DNA fragment was amplified by PCR and cloned into the pTAdvance vector (Clontech). The sequence of the cloned IL-1β 3'UTR was verified by automated sequencing.

To obtain the regulatory elements present in the **TNF-α 3'UTR** PCR primers based on the TNF-α genomic sequence (U06950) were designed to encompass the entire TNF-α 3'UTR including the poly-adenylation signal. Using these primers, a 994bp long DNA fragment amplified by PCR was cloned into the pTAdvance vector. The sequence of the cloned TNF-α 3'UTR was verified by automated sequencing.

To obtain the regulatory elements present in the **GAPDH 3'UTR**, PCR primers based on GAPDH genomic sequence (M32599) were designed to encompass the entire GAPDH 3'UTR including the polyadenylation signal. Using these primers, a 184bp long DNA fragment amplified by PCR was cloned into the pTAdvance vector. The sequence of the cloned GAPDH 3'UTR was verified by automated sequencing.

Generation of the second series of reporter constructs

The second series of reporter constructs consists of a number of plasmids where the expression of the reporter gene is under the regulation of promoter sequences and 3'UTRs of cytokines: IL-4, IL-2, IFN-γ, IL-1β, and TNF-α. Reporter constructs are based on the plasmids pEGFP-1, pEGFP-F, and pd1EGFP-N1, pd2EGFP-1 (Clontech), containing enhanced green fluorescent protein (EGFP) and destabilized enhanced green fluorescent protein (dEGFP) as a reporter gene, respectively.

To obtain EGFP expression vector in which 3' downstream regulatory sequences of **IL-4** gene control the mRNA stability of the EGFP transcript, the pTAdvance vector containing the entire 3'UTR for IL-4 was digested with EcoRI (NEB) and the obtained fragment was ligated downstream of EGFP stop codon into the pEGFP-F vector using the EcoRI site. The presence of the IL-4 3'UTR was confirmed by an EcoRI digest and by PCR. The right orientation of the IL-4 3'UTR was confirmed by ScaI and BsmI (NEB) digests. Finally, the sequence was confirmed by automated sequencing. The constructed vector, named **p2-3'IL4/EGFP-F**, contains SV40 derived 3'UTR sequences including polyadenylation sites. Northern blot experiments showed that these polyadenylation sites were utilized during transcription process. Thus, in the next step, the p2-3'IL4/EGFP-F vector was modified by removing the interfering SV40 polyadenylation signals. This plasmid was digested with BsmI (NEB) and Bal-31 nuclease (NEB), re-ligated and transformed into *E. coli* cells. Following

plasmid isolation, the absence of the sequence containing SV40 polyadenylation signals in the modified plasmid, named **p3-3'IL4/EGFP-F**, was confirmed by a BsmI digest and sequencing. In the next step, IL-4 3'UTR was excised from pTAdvance plasmid with EagI (NEB) and ligated into NotI (NEB) site downstream of EGFP stop codon in the pd1EGFP-N1 vector. Insert presence was confirmed by PCR and an EagI digest, and orientation was checked with a PstI (MBI Fermentas) digest. The sequence of the obtained plasmid, named **p1-3'IL4/d1EGFP-N1,** was confirmed by sequencing. To obtain the EGFP expression vector in which both 5' upstream and 3' downstream regulatory sequences of IL-4 gene control the expression of the reporter gene, vector p1-5'IL4/d1EGFP-N1, containing IL-4 promoter and d1EGFP ORF and vector p1-3'IL4/d1EGFP-N1 containing d1EGFP ORF and IL-4 3'UTR were both digested with AgeI (MBI Fermentas) and HincII (NEB) enzymes. DNA fragment containing d1EGFP ORF and IL-4 3'UTR obtained from p1-3'IL4/d1EGFP-N1 digest was next ligated with DNA fragment containing vector sequence and IL-4 promoter derived from p1-5'IL4/d1EGFP-N1. The restriction map of the new construct, named **p1-5'3'IL4/EGFP-N1**, was verified by digestion with Eco52I (MBI Fermentas), followed by sequencing.

To obtain EGFP expression vectors in which both 5' upstream and 3' downstream regulatory sequences of **IL-2** and **INF-γ** genes control the expression of the reporter gene the following strategy was used. First, the respective 3'UTRs were cloned into the pd2EGFP-1 vector. To this end the SV40 derived polyadenylation signals present in the pd2EGFP-1 vector were removed using the restriction enzymes NotI and AflII. The 3'UTR's were then excised from the PCR cloning vector pGEM-T Easy using the restriction enzymes NotI and AflII, and the 3'UTRs containing fragments were ligated into NotI/AflII restricted pd2EGFP-1 vector. In the next step, the IL-2 and IFN-γ promoter regions were cut out from the plasmids p1-5'IL2/EGFP-1 and p1-5'INFγ/EGFP-1, respectively, using the restriction enzymes XhoI and BamHI. These fragments were then gel-purified and ligated into the pd2EGFP-1 vectors containing 3'UTRs using XhoI and BamHI sites in the multiple cloning site upstream of d2 EGFP gene. Restriction maps of the new constructs, named **p1-5'3'IL2/d2EGFP-1** and **p1-5'3'INFγ/d2EGFP-1,** were verified by multiple restriction digests and sequencing.

Two EGFP expression vectors in which both 5' upstream and 3' downstream regulatory sequences of **IL-1β** control the expression of the reporter gene were constructed. IL-1β 3'UTR was excised from pTAdvance vector with Eco52I (MBI Fermentas) and ligated into p1-5'IL1β/d1EGFP-N1 and p3-5'IL1β/d1EGFP-N1 vectors digested with NotI (NEB). The presence of the IL-1β 3'UTR in both plasmids was confirmed by PCR and its orientation was checked by HincII (NEB) digest. Sequences of new vectors, named **p1-5'3'IL1β/d1EGFP-N1** and **p3-5'3'IL1β/d1EGFP-N1,** were confirmed by sequencing.

To obtain the EGFP expression vector in which 3' downstream regulatory sequences of **TNF-α** gene control the mRNA stability of the EGFP transcript, the pTAdvance vector containing the entire 3'UTR for TNF-α was digested with HindIII and EcoRV (NEB) and the obtained fragment was ligated downstream of EGFP stop codon into pEGFP-F vector using sites HindIII and SmaI. The presence of the TNF-α 3'UTR was confirmed by EcoRI and SspI (NEB) digest and by PCR. The sequence of TNF-α 3'UTR was confirmed by automated sequencing. Resultant vector, named **p2-3'TNFα/EGFP-F**, contains SV-40 derived 3'UTR sequences including polyadenylation sites. As Northern blot experiments conducted with p2-3'IL4/EGFP-F plasmid showed that SV-40 derived polyadenylation sites are utilized during transcription process the p2-3'TNFα/EGFP-F vector was modified by removing SV40 polyadenylation sequences. Plasmid p3-3'IL4/EGFP-F was digested with AseI and HincII to remove CMV-GFP-3'UTR IL-4 box and ligated with CMV-GFP-3'UTR THF-α box cut out from the plasmid p2-3'TNFα/EGFP-F using AseI and HincII. The presence of TNF-α 3'UTR in the resulting plasmid, named **p3-3'TNFα/EGFP-F,** was confirmed by PCR and the absence of SV40 polyadenylation sites by BsmI digest. In the next step, TNF-α 3'UTR was excised from pTAdvance plasmid with EagI (NEB) and ligated into the NotI (NEB) site downstream of EGFP stop codon into the pd1EGFP-N1 vector. Insert presence was confirmed by PCR and by Eco52I digest, and the right orientation of TNF-α 3'UTR was confirmed BglII and Bsu36I (MBI Fermentas) digest. The sequence of the constructed **p1-3'TNFα/d1EGFP-N1** plasmid was confirmed by sequencing. To obtain EGFP expression vector in which both 5' upstream and 3' downstream regulatory sequences of **TNF-α** control the expression of the reporter gene the following strategy was used. First, SV40 3'UTR from p1-5'TNFα/d1EGFP-N1 plasmid

was removed by sequential digestions with the following enzymes: Mva1269I (MBI fermentas), Mung Bean nuclease (NEB) and NotI (NEB). The 6684 bp vector fragment without SV40 3'UTR was next ligated with the 1039 bp fragment containing TNF-α 3'UTR excised from the p1-3'TNFα/d1EGFP-N1 using NotI and EcoRV endonucleases. The integrity of constructed vector named p1-5'3'TNFα/d1EGFP-N1 was confirmed by sequencing.

To obtain the EGFP expression vector in which 3' downstream regulatory sequences of **GAPDH** (the control house-keeping gene) control the mRNA stability of the EGFP transcript, the pTAdvance vector containing the entire 3'UTR for GAPDH was digested with EcoRI (NEB) and the obtained fragment was ligated downstream of EGFP stop codon into pEGFP-F vector (Clontech) using the EcoRI site. The presence of the GAPDH 3'UTR was confirmed by EcoRI digest and by PCR. The right orientation of the GAPDH 3'UTR was confirmed by StyI (NEB) digest. Finally, the sequence of the construct was confirmed by automated sequencing. The resulting vector, named **p2-3'GAPDH/EGFP-F**, contains SV40 derived 3'UTR sequences, including polyadenylation sites. As Northern blot experiments conducted with p2-3'IL4/EGFP-F plasmid showed that SV-40 derived polyadenylation sites are utilized during transcription process, the p2-3'GAPDH/EGFP-F vector was modified by removing SV40 polyadenylation sequences. Plasmid p3-3'IL4/EGFP-F was digested with AseI and HincII to remove CMV-GFP-3'UTR IL-4 cassette and ligated with CMV-GFP-3'UTR GAPDH cassette cut out from the plasmid p2-3'GAPDH/EGFP-F using AseI and HincII. The presence of GAPDH 3'UTR in the resulting plasmid named **p3-3'GAPDH/EGFP-F** was confirmed by PCR and the absence of SV40 polyadenylation sites by BsmI digest. In the next step, GAPDH 3'UTR was excised from the pTAdvance plasmid with EagI (NEB) and ligated into the NotI (NEB) site downstream of the EGFP stop codon into the pd1EGFP-N1 vector (Clontech). The presence of the insert was confirmed by PCR and by Eco52I digest, and the right orientation of GAPDH 3'UTR was confirmed PstI (MBI Fermentas) digest. The sequence of the resultant **p1-3'GAPDH/d1EGFP-N1** plasmid was confirmed by sequencing.

Consequently, a set of expression vectors was obtained, in which the GFP coding sequence is under the control of 5' upstream regulatory sequences derived from cytokine gene, in a form of highly purified DNA available for transfection of mammalian cells. Moreover, a set of

expression vectors was obtained, in which the GFP coding sequence is under the control of 5' upstream and 3'downstream regulatory sequences derived from cytokine genes, in a the form of highly purified DNA available for transfection of mammalian cells.

Example 2. Cloning of the first set of reporter cell lines

To obtain several cell lines (The First Generation of Reporter Cell Lines) of desirable phenotype for testing immunotoxicity by introduction of the reporter gene into existing immortalized cells under the control of cytokine gene-derived regulatory DNA sequences.

Methodology and study materials

Work started with the transfection of selected cell lines with the expression vectors obtained in Example 1. T cells and T cell hybridoma, mast cell lines, monocyte-macrophage, fibroblasts and keratinocyte cell lines were used. Plasmids were linearized by digestion and cells were transfected using electroporation.

Given cell lines were transfected with GFP constructs corresponding to cytokine genes known to be expressed at a relatively high level by this cell line upon activation. Transfectants were selected in culture by selective media (G-418) and cellular clones were developed by limiting dilution cloning. Clones were expanded and tested with RT-PCR for the presence of vector derived sequences. Positive clones underwent another round of cloning followed by screening with RT-PCR. Resultant clones were expanded in large-scale cultures and multiple aliquots were frozen. Cell lines obtained at this stage were used for further testing and development of stable cell lines. Several characteristics of developed cell lines were determined, such as the expression of cell surface markers and lineage specific functional responses (CD3 dependent proliferation, IgE-mediated degranulation, LPS induced phagocytosis). Next, immunological and pharmacological stimuli were used to activate cells and measure their GFP expression, and endogenous cytokine expression. This reveals whether there is a parallel activation of reporter gene and a cytokine gene of interest. Next the cytotoxicity test compatible with the fluorescence assays was developed. This is necessary to monitor tested compounds for frank cytotoxicity. For that purpose flourescent dyes based a cell viability assay, measurements of autoflorescence and the level of flourescence associated with expression of control GFP construct were tested. These experiments resulted in the development of the experimental

protocol that would be used in Example 7 to measure kinetics of cell viability and cytokine expression inducing signal in parallel.

Consequently, some of the cell lines tested in Example 7, were used to assemble the panel of reporter cell line i.e."a prototype of a cell-chip".

Development of an assay for cell viability compatible with GFP detection

Three viability assays have been chosen for preliminary tests: NRU (Neutral Red Uptake), NRR (Neutral Red Release), and MTT (MTT Assay). In these assays **3T3-L1** fibroblasts and two immunotoxic compounds **$HgCl_2$** and **SDS** (two of the chemicals from the list of model immunotoxins in the Table II of the Technical Annex) were used. As a result, the effective concentration of $HgCl_2$ and SDS that leads to death of 50% of cells in the population (EC50) were determined.

Organisation of the cell line banking system

For storage of the cell clones generated during the project a distinct room in the NIOM facility was assigned. To assure the free space needed for cell samples additional liquid nitrogen cryogenic storage vessels were arranged. The standard protocol for collection and freezing of samples was elaborated. The control system of sample banking was prepared in details.

Optimization of protocols for stable transfection and EGFP detection

Several protocols for stable transfection have been used to generate reporter cell lines.

For adherent cell lines the transfection technique using lipofectamine was employed. In a standard experimental protocol, 2 x 10^5 cells seeded in a 24-well plate in complete DME medium (Sigma) were washed with serum free medium and incubated for about 2h in 37°C, 5% CO_2. Next, DNA with lipofectamine (Gibco) was added and cells were incubated for another 5h. Following this incubation medium containing 20% FCS was added and cells were cultured for 24h. This medium was then replaced with a medium containing 10% FCS. 72h after transfection, selecting medium containing G-418 (Gibco) at a concentration appropriate for a given cell line was added and cells were cultured for about 7 days. Limiting dilution was used next to clone the transgene positive cells. Expression of GFP was verified by FACS and using fluorescent microscope.

For cell lines which grow in suspension, the electroporation based transfection technique was employed. In a standard experimental protocol 1 x 10^7 cells grown in an appropriate medium were electroporated with 20 to 50 μg of DNA. Cells were allowed to recover in normal growth medium for 48 hours before the selecting antibiotic (G-418) was added. Next, the cells were either directly cloned by limiting dilution, or were first cultured in selecting medium for additional 1-2 weeks and then cloned. Resistant clones were expanded, frozen and characterized phenotypically.

To test the transfection protocol and techniques for GFP detection easily transfectable human T cell line Jurkat was used. Jurkat cells were transfected with the pEGFP-F plasmid where EGFP expression is driven by CMV promoter. As a result several cell clones with a high expression of EGFP were obtained. These cell lines were used by DBAPAS to test EGFP expression with a phosphorimager Multimager Typhoon 8600 (Molecular Dynamics).

Cloning of the first set of reporter cell lines

To obtain several cell lines of desirable phenotype for testing immunotoxicity with the reporter gene under the control of cytokine gene-derived regulatory DNA sequences several cell lines listed in the Table I of the Technical Annex were transfected with reporter GFP constructs. First, cells were transfected with plasmids, in which EGFP is under the control of a strong viral promoter (CMV). Thus, C57.1 mast cell line and HEL-30 keratinocytes were transfected with pEGFP-F plasmid, and 3T3-L1 fibroblasts were transfected with pEGFP-F and pEGFP-N1 plasmids. EL4 and BW5147.3 lymphocytes were transfected with pEGFP-C3 plasmid. As a result, several cell lines expressing different levels of EGFP have been generated, and are used to characterize the effects of stable transfection and EGFP expression on the cell line phenotype. In one of these transfection experiments, monitoring of GFP expression with Multimager was applied at early stages of cloning of 3T3-L1 fibroblast transfected with pEGFP-F. The resultant fibroblast cell line T/pEGFP-F/1 was used to develop alternative GFP detection technique (see WP.7). C57.1 mast cells expressing EGFP under control of the CMV promoter were employed in a series of experiments to assess the effect of stable EGFP transfection on their morphological and functional features. These cells expressed EGFP at a level easily detectable by FACS or under the fluorescent microscope. C57/CMV-

EGFP cells shoed morphology identical to the maternal cell line. An increase in the number of giant cells as compared to the maternal cell line was observed. When tested in functional assays these cells exhibited the normal characteristics of mast cells. They were sensitized with monoclonal IgE *in vitro* and responded to an antigen by exocytosis and cytokine production. The level of mediator release and cytokine production in C57/CMV-EGFP cells were comparable to those observed with maternal C57.1 mast cells.

Next, DNA constructs developed in Example 1, in which GFP expression was under the control of 5' upstream or 3' UTR regulatory sequences of cytokine genes were used. Thus, C57.1 mast cells were transfected with the EGFP reporter plasmid for IL-4 gene, p1-5'IL4/EGFP-1. The plasmid was linearized and cells were transfected using electroporation. Several neomycin resistant clones were obtained and were expanded in large-scale cultures. Following second round of cloning one of these clones has been found to have a proper phenotypic characteristic of mast cells and detectable EGFP expression. This resultant cell line in which IL-4 minimal promoter controls EGFP expression was named C57/5'IL4/1 (renamed to C/p1-5'IL4-EGFP/002, in accordance with the labeling system in the reporter cell line database) and is the M1 milestone of the project. This cell line was employed in a series of experiments testing the level of EGFP expression in resting cells and cells challenged with ionophore. The conclusion of these tests was that there was a high EGFP expression in resting cells, which was due to the high stability of the EGFP protein. This feature in turn seems to obscure the increase in EGFP expression following cell stimulation. Based on this data the conclusion was drawn to also employ the destabilized EGFP variant, called dEGFP. in addition to EGFP (see Example 3).

Lymphocytic cell lines EL4 and BW5147.3 were transfected with reporter constructs for IL-2 and IFN-γ, p1-5'INFγ/EGFP-1 and p1-5'IL2/EGFP-1, respectively. Several resistant clones resulting from these transfections have already been frozen. EL4 cells transfected with the p1-5'IL2/EGFP-1 plasmid were used to test spontaneous and phorbol ester mediated expression of EGFP mRNA. For each type of reporter cell line a minimum of 3 clones have been obtained. The clones were initially analyzed on the basis of GFP basal expression and inducibility. Initial analysis was performed using fluorescence microscopy, demonstrating this to be a good

method to detect GFP expression. Attempts to use Fluorescence microscope reader resulted in the need for further optimization of experimental protocols and technical details. Subsequent functional analyses have been performed using flow cytometry (FACS). Basal GFP expression relative to non-transfected cells, as well as inducibility of expression after athe ctivation of cells with TPA+ionomycin was tested. Regarding the EL4 derived clones with GFP under control of regulatory elements from IL2 or IFNγ, several clones with low basal and high inducible GFP expression were identified. Regarding the BW5147.3 derived clones, all tested clones show a detectable basal GFP expression but none of the clones showed an increased expression of GFP following activation with TPA and ionomycin.

Lymphocytic cell line EL4 was transfected with a reporter construct for IL-4 p1-5'IL-4/EGFP-1. Multiple resistant clones resulting from these transfections have been tested using FACS and fluorescence microscopy for basal and inducible EGFP expression. None of the clones showed an increased expression of GFP following activation with TPA and calcium ionophore.

Consequently, the set of reporter cell lines with a proper phenotypic characteristic containing reporter genes incorporated into chromosomal DNA was obtained. Moreover, the assay for cell viability compatible with the fluorescence assay for GFP expression was obtained.

Example 3. Construction of modified expression vectors for stable transfections

To modify the DNA constructs in which the expression of reporter fluorescent protein depends on regulatory sequences derived from cytokine genes by introduction of additional regulatory sequences and/or modification of fluorescence protein coding sequences. These modifications shall result in an increase in the ratio of maximal to baseline expression of reporter gene. This objective was reached by one or several of the following changes: Lowering the spontaneous baseline expression of fluorescent protein; (and/or) increasing the amount of reporter protein expressed upon stimulation; (and/or) changing the cellular localization of fluorescent protein; (and/or) changing the type of fluorescent protein. These modified expression vectors (Modified Expression Vectors for Stable Transfection) are tools for genetic modification of cell lines and will be used in Example 4.

Methodology and study materials

The GFP constructs obtained in Example 1 were modified using standard molecular biology techniques, by restriction enzyme digestion and directional cloning of desired sequences. The sequences of interest were either derived from commercially available expression vectors or were introduced as synthetic oligonucleotides. Positive clones were selected and the sequence of the obtaining plasmids verified. The detailed changes in the design of expression vectors depend of the results obtained. Lowering of the baseline level of GFP expression may require the introduction of additional regulatory elements such as silencers into the upstream of GFP coding sequence. Additional "heterologous" motif destabilizing mRNA can be also introduced into the 3' UTR downstream of GFP open reading frame.

To increase the level of expression the repeated tandem regulatory sequences of cytokine promoter can be used and known "heterologous" motifs stabilizing mRNA can also be introduced into the 3' UTR downstream of GFP open reading frame.

The ladder sequences in the GFP open reading frame can be modified resulting into different trafficking of GFP into cellular compartment. The fluorescent protein itself can be replaced with one of several modified fluorescent proteins, which may change the signal to noise ratio in fluorimetric readout of gene expression due to different level of overlap with autofluorescence. The bioluminescence based reporter gene can also be tested as a possible alternative for GFP system.

All this modification were obtained by changing the existing expression vectors using standard molecular biology techniques, such as restriction enzyme digestion, PCR and ligation.

Generation of the dEGFP reporter constructs containing cytokine 5' regulatory regions

Two reporter constructs containing dEGFP under control of ***IL-4*** 5' upstream regulatory sequences were created. The first one was generated based on the p1-5'IL4/EGFP-1 plasmid (see WP1) digested with SmaI and XhoI enzymes (MBI Fermentas). The excised fragment containing minimal promoter for *IL-4* (-87/+5), was cloned into the the pd1EGFP-N1* plasmid (pd1EGFP-N1 modified by removal of CMV promoter, see WP1) digested with the same pair of enzymes. Ligation products were screened with SmaI and Alw44I digestion. The sequence of constructed plasmid, named **p1-5'IL4/d1EGFP-N1,** was confirmed by automated

sequencing. For the second reporter construct, a longer *IL-4* promoter region (-797/+5) was obtained by PCR (Taq Polymerase; MBI Fermentas) using the -797pCAT plasmid as a template (a gift from Dr. Melisa Brown, Atlanta University, Atlanta, USA). PCR product was cloned into pTAdvance vector. Next, *IL-4* promoter was excised from the pTAdvance vector with SmaI and VspI (MBI Fermentas) and cloned into pd1EGFP-N1* digested with the same pair of enzymes. The effect of the ligation was confirmed by HindII and by Eco52I digestions. The sequence of the constructed plasmid, named **p2-5'IL4/d1EGFP-N1,** was verified by sequencing.

Reporter constructs containing dEGFP under control of ***IL-2*** and ***IFN-γ*** 5' upstream regulatory sequences are based on reporter constructs containing EGFP. The IL-2 and IFN-γ promoter regions were excised using XhoI and BamHI from plasmids p1-5'IL2/EGFP-1 and p1-5'INFγ/EGFP-1, respectively. The obtained DNA fragments were then gel-purified and ligated into the pd2EGFP-1 plasmid (Clontech), digested with the same pair of enzymes. Ligation products were analysed using multiple restriction digests. Sequences of the obtained constructs, named **p1-5'IL2/d2EGFP-1** and **p1-5'INFγ/d2EGFP-1,** were confirmed by sequencing.

Two improved reporter constructs containing dEGFP under control of ***IL-1β*** 5' upstream regulatory sequences were created. The rationale was to obtain reporter constructs with intronic sequences of ***IL-1β***, which are thought to possess regulatory capacity. The PCR primers were designed to encompass the sequence from –4093 bp or –500 bp upstream of transcription start to the beginning of exon 2 (+820 bp) of the *IL-1β* gene. –500/+820 and –4093/+820 fragments of *IL-1β* obtained by PCR using Balb/c mouse genomic DNA as a template and a high-fidelity AccuTaq polymerase (Sigma) were cloned into pCR-Blunt II-TOPO vector (Invitrogen). The *IL-1β* derived sequences were then released from this vector using EcoRV (NEB) and KpnI (NEB) and cloned into pd1EGFP-N1* digested with SmaI (MBI Fermentas) and KpnI (NEB). The presence of the –500/+820 *IL-1β* promoter in the first obtained plasmid, named **p2-5'IL1β/d1EGFP-N1**, was confirmed by PCR and HincII digest. The presence of the –4093/+820 *IL-1β* promoter in the second obtained plasmid, named **p4-5'IL1β/d1EGFP-N1**, was confirmed by PCR, and by digestion with Eco88I (MBI Fermentas).

Sequences of the **p2-5'IL1β/d1EGFP-N1** and **p4-5'IL1β/d1EGFP-N1** plasmids were confirmed by automated sequencing.

To obtain dEGFP reporter construct under control of actin promoter, CAG promoter, which consists of CMV enhancer and chicken β-actin proximal promoter was cut out with AseI and Eco47III restriction enzymes from commercially available pQE-TriSystem vector (Qiagen) and ligated into pd1EGFP-N1vector (Clontech) in the place of CMV promoter (cut out with AseI and Eco47III restriction enzymes), upstream of EGFP coding sequence. The sequence of resultant construct **pCA-d1EGFP** was confirmed by restriction enzyme maping.

Generation of the dEGFP reporter constructs containing cytokine 5' and 3 regulatory regions

To obtain dEGFP reporter construct containing both, the -87 promoter and 3'UTR of ***IL-4*** gene, the plasmids p1-5'IL4/d1EGFP-N1 and p1-3'IL4/d1EGFP-N1 were digested with AgeI (MBIFermentas) and HincII (NEB) The fragment containing d1EGFP and 3'UTR excised from p1-3'IL4/d1EGFP-N1 plasmid was next cloned into p1-5'IL4/d1EGFP-N1, immediately downstream of *IL-4* promoter. A restriction map of a new construct, named **p1-5'3'IL4/d1EGFP-N1**, was analysed by Eco52I (MBI Fermentas) digestion and the sequence of this plasmid was verified by automated sequencing. To obtain dEGFP reporter construct containing both, the -797 promoter and 3'UTR of ***IL-4*** gene, the plasmids p2-5'IL4/d1EGFP-N1 and p1-3'IL4/d1EGFP-N1 were digested with AgeI (MBI Fermentas) and HincII (NEB). The fragment containing d1EGFP and 3'UTR excised from p1-3'IL4/d1EGFP-N1 plasmid was next cloned into p2-5'IL4/d1EGFP-N1, immediately downstream of *IL-4* promoter. A restriction map of the new construct, named **p2-5'3'IL4/d1EGFP-N1**, was analysed by Eco52I and SspI (NEB) digestion and the sequence of this plasmid was verified by automated sequencing.

To obtain dEGFP reporter construct containing both, the –500/+820 5' regulatory region and 3'UTR of ***IL1β*** gene, the *IL-1β* 3'UTR was excised from pTAdvance with EagI (NEB), and ligated into p2-5'IL1β/d1EGFP-N1 digested with NotI (NEB). The presence of the insert was confirmed by PCR and its orientation was analysed by Ecl136II (MBI Fermentas) digestion. The sequence of the obtained plasmid, named **p2-5'3'IL1β/d1EGFP-N1**, was confirmed by

automated sequencing. To obtain the dEGFP reporter construct containing both, the –4093/+820 5' regulatory region and 3'UTR of *IL-1β* gene, the *IL-1β* 3'UTR was excised from pTAdvance with EagI (NEB), and ligated into p4-5'IL1β/d1EGFP-N1 digested with NotI (NEB). The presence of the insert was confirmed by PCR and its orientation was determined by an Ecl136 (MBI Fermentas) digest. The sequence of the obtained plasmid, named **p4-5'3'IL1β/d1EGFP-N1**, was confirmed by automated sequencing.

Consequently, a set of modified expression vectors was obtained, in the form of highly purified plasmid DNA available for transfection of mammalian cells.

Example 4. Cloning of the improved reporter cell lines

To obtain several cell lines (The Second Generation of Reporter Cell Lines) of phenotype characteristics improved as compared to the first generation of reporter cell lines for testing immunotoxicity.

Methodology and study materials

Work will start with transfection of selected cell lines using the GFP expression vectors modified as described in Example 3. Linearized plasmid DNA will be employed to transfect cells using electroporation. Transfectants that underwent random insertion of extrachromosomal DNA will be selected by culture in the presence of G-418 and cloned with limited dilution cell cloning. Clones will be expanded, verified for the presence of vector DNA with PCR, and expanded in large-scale cultures. Multiple aliquots will be frozen. Next, all the important phenotype characteristics of these cell lines will be investigated using a similar approach to that described in Example 2. The level of GFP expression and the signal to noise ratio will be determined. These data will be compared to the parameters observed in the cell lines obtained in Example 2. It will show whether the modifications of the GFP reporter gene constructs will improve the detection of inducible gene expression using fluorimetric or luminometric assays. It is desirable to clone and characterize 3 to 4 cell lines transfected with modified GFP expression vectors. This number of cell lines should be sufficient for the verification of our assumptions. Some of these cell lines will be used in Example 7 to build the panel of reporter cell lines "a prototype of a cell chip".

Results

To obtain several cell lines of phenotype characteristics improved as compared to the first generation of reporter cell lines for testing immunotoxicity the lymphocytic cell lines **EL4** and **BW5147.3** were transfected with dEGFP reporter constructs for *IL-2* and *IFN-γ*, **p1-5'IL2/d2EGFP-1** and **p1-5'INFγ/d2EGFP-1**, respectively (Example 3). Several neomycin resistant clones resulting from these transfections were expanded and characterized. Some of them have shown low basal dEGFP expression, which was upregulated upon activation with TPA/ionomycin to the level easily detected by FACS or fluorescence microscopy. In such a manner, the reporter cell line EL/p1-5'IL2-dEGFP/7 was obtained. Although clones carrying destabilized variant of EGFP show lower basal expression when compared to EGFP transfected cells the maximum level of inducible dEGFP expression was also lower as compared to activated EL4 clones carrying dEGFP transgene. Lymphocytic cell line EL4 was also transfected with a reporter constructs for IL-4 p1-5'IL4/d1EGFP-N1 and p1-5'3'IL4/d1EGFP-N1. These transfections resulted in multiple neomycin resistant clones, which were tested for basal and inducible EGFP expression using FACS and fluorescence microscopy. None of the clones showed an increased expression of GFP following activation with TPA and calcium ionophore.

C57.1 mast cells were transfected with reporter constructs for *IL-4* **p1-5'IL4/d1EGFP-N1** and **p1-5'3'IL4/d1EGFP-N1**. Resultant clones were characterized for basal and inducible GFP expression and did not show induction of GFP expression. The transfection protocol using the same plasmids was repeated and generated multiple resistant clones.

J774.1A monocytes-macrophages were transfected with the reporter constructs for TNF-α, **p1-5'TNF-α/d1EGFP-N1**. Multiple resistant clones were selected and cloned. Resultant clones were characterized for basal and inducible GFP expression using FACS. One of these clones was found to respond to activation with LPS by upregulation of GFP fluorescence.

C57.1 mast cells were transfected with two reporter constructs for TNF-α, **p1-5'TNF-α/d1EGFP-N1** and **p1-5'TNF-α/d1EGFP-N1.** Multiple resistant clones were selected and cloned. Resultant clones were characterized for basal and inducible GFP expression using fluorescence microscopy and FACS. 10 of these clones have been found to respond to

activation with TPA and A23 ionophore by upregulation of GFP fluorescence, easily detected under fluorescence microscopy and FACS.

EL4 cells and C57 cells were transfected control plasmid pCA-d1EGFP, in which dEGFP expression is under control of the actin promoter. Resultant cell clones were tested for GFP expression and showed a basal expression that is not inducible upon activation.

3T3-L1 fibroblasts, Hel-30 keratinocytes, and J774.1A monocytes-macrophages were transfected with the IL-1β reporter constructs **p2-5'IL1β/d1EGFP-N1** and **p4-5'IL1β/d1EGFP-N1**. HEL-30 derived resistant cells were found to be difficult to clone and propagate due to very strong adhesion to plastic. An alternative technique for transfection of this cell line is currently being tested. Transfection of J774A.1 yielded multiple resistant clones that were characterized for selected phenotypic features and GFP expression. Analysis of cell size performed on a Coulter Multisizer II confirmed observations conducted on these cell lines under microscope, which suggested a greater cell size for the original J774A.1 line. The clones together with the original line were stimulated with LPS (1 μg/ml) and assessed for: NO production using the Griess reaction, IL-1β protein synthesis (using DuoSet ELISA Development kit (R&D), GFP and IL-1β mRNA expression (RT-PCR using RevertAidTM (Invitrogen), and GFP fluorescence with a fluorescence microscope equipped with CCD camera.

Data from the nitric oxide production assay suggested that LPS activated both the nontransfected J744 A.1 cells and all tested clones to similar extent. IL-1β protein synthesis measurements showed no detectable concentrations in the supernatants of stimulated cells but high quantities of IL-1β in cell lysates. All stimulated clones (in 25 cm^2 culture flasks, 70-80% confluency) expressed mRNA for GFP, which was associated by simultaneous IL-1β mRNA expression. GFP expression was observed after 6 and 12h of LPS stimulation and then slowly declined on 24^{th} hour. Interestingly however, only two among these five clones showed increase in GFP fluorescence following stimulation.

Transfection of **3T3-L1** yielded multiple resistant clones that were characterized for basal and inducible GFP expression. 40 resultant clones were tested for their response to stimulation

with LPS using FACS. Two of these clones responded to LPS by upregulation of GFP fluorescence.

Example 5. Construction of expression vectors for gene targeting

To prepare a series of DNA vectors designed for gene targeting. These targeting vectors are tools necessary for development of immune cells (Example 6) in which selected loci of cytokine genes will be replaced with a reporter gene.

Methodology and study materials

Gene-targeting strategy utilizing a targeting vector with a long contiguous sequence homologous to the targeted loci will be used. First, several clones of genomic DNA will be collected, overlapping or mapped close to murine cytokine genes. The restriction map of the relevant part of murine chromosomal DNA covering loci for IL-4, IL-5, and IFN-γ are available, and these genes were successfully targeted in mice resulting in a "knockout phenotype". The gene targeting constructs will contain a 6 kB fragment of DNA overlapping the coding region of targeted cytokine gene. For each targeted locus it is desirable to prepare three vectors, each of them containing the same long homologous sequence but differ in the type of selectable markers. Construction of targeting vectors will start based on a backbone of a standard pBluescript vector. GFP coding region with 3'UTR cytokine sequences adjacent to its Stop codon will be derived from GFP vectors obtained in Example 1. In the first type of vectors, GFP coding sequences followed by 3'UTR of cytokine genes will be inserted into the 6 kB long fragment homologous sequence to replace the cytokine open reading frame. A Neo or Hyg box flanked with a pair of loxP sequences will be inserted downstream of the transcription termination signal for the cytokine gene followed by long contiguous fragment of targeting homologous sequence. Inserting a tk gene close to the end of the 3kB long downstream homologous sequence will develop the second type of targeting vector, which allows the use of the positive-negative cell selection technique (PNS). Vectors will be constructed using standard DNA manipulation techniques, including restriction enzyme digestion and ligation. The synthetic oligonucleotide adapters and PCR generated DNA fragments will be used if necessary to connect the desired DNA sequences and to introduce particular sequences into the construct. Following transformation the positive *E coli* clones

will be selected with a miniprep analysis. After verification of plasmid sequences with automated sequencing, large quantities of plasmid DNA will be amplified and purified.

Results

Experimental work exploring the gene-targeting approach for development of reporter cell lines started by using the 9 kb IL-2/GFP targeting construct obtained from Dr. Hua Gu (Laboratory of Immunology, NIH Rockville, USA). This plasmid, containing the 2 kb sequence that encompasses *IL-2* upstream region, GFO ORF from pGgreenLantern (Life Technologies), Neo box and 4 kb of *IL-2* genomic DNA, had been successfully applied for generation of transgenic mice (Immunity 9: 209-216). The integrity of this vector has been verified, amplified and purified plasmid DNA and employed in a series of experiments. The original plasmid is modified by addition of TK box at the 3' end, which allows the use the positive-negative cell selection technique (PNS). To this end the pPNT plasmid containing the herpes simplex virus thymidine kinase gene under control of the mouse phosphoglycerate kinase-1 promoter (BCCM/LMBP plasmid and DNA collection, Ghent, Belgium) was used. An extensive restriction mapping was performed to identify sites in the pIL-2/GFP plasmid that could be used to clone in the TK-cassette. Only a few single cutters that did not disrupt the IL2/GFP targeting sequence were identified. These restriction sites does not correspond to any available in the pPNT plasmid. To overcome the lack of sites in the targeting construct the 2.8 kb TK cassette was sub-cloned into pBluescript. An alternative PCR based cloning strategy was also tested.

Censequently, it is desirable to obtain a set of gene targeting vectors, containing long fragments of DNA overlapping the coding region of a targeted cytokine, GFP coding sequence and selectable marker or markers, in the form of highly purified DNA available for transfection of mammalian cells.

Example 6. Exploring the gene targeting technology for generation of reporter cell lines

To engineer immortalized immune cells, in which signals regulating transient expression of cytokine gene would instead regulate the expression of reporter gene. Thus, the entire complexity of regulatory mechanisms controlling cytokine production with all *cis* and *trans* acting elements would influence the level of GFP expression.

Methodology and study materials

Vectors obtained in Example 5 will be used to target DNA sequences into the selected cytokine gene loci. Vectors designed for positive selection with neomycin (G418) or hygromycin and vectors for positive-negative selection (PNS) will be used. Cutting at the unique restriction enzyme site will linearize the vectors, which will be then electroporated into cells. Cells will be placed in selecting media. Selection of cells will be based on G418 or hygromycin in case of positive selection process or on G418 followed with gancyclovir in a positive-negative selection process. Resistant cells will be cloned by limiting dilution procedure. Clones will be screened for desired genomic modification using PCR. The selected clones will be further tested for successful gene targeting using southern blot, and expanded in large-scale cultures. Cell lines obtained at this stage will undergo tests for phenotypic characteristics. In the next step it will be desirable to activate these cells and measure GFP expression. The successful incorporation of a transgenic insert into the specific loci will result in a single allele modified to express GFP instead of cytokine mRNA. In the next step it will be desirable to replace both alleles of the gene with GFP and compare the resulting phenotype with that of a single replacement. To do this, cell lines with single transgenic insertions and which target the other allele will be used. One possible strategy is to use the targeting vector with different selectable markers and to screen transfectants for double resistance. An alternative strategy is to remove the neomycin cassette from the transgenic insert and use the neomycin resistance marker for selection once again. Clones will be tested using PCR and clones negative for the neo box and sensitive to neomycin will be selected. From this point the second round of gene targeting is similar for both strategies described above and follows the experimental procedure described for the first gene targeting experiment. The difference will be used for screening in the PCR primers and Southern probe. They will be designed to verify the complete absence of cytokine coding sequences in the genomic DNA.

Results

The 9 kb IL-2/GFP targetting construct was linearized and used for electroporation-based transfection of EL4 cells. Transfected cells underwent G-418 based selection. Limited dilution cloning of neomycin resistant cells yielded 26 clones, which were characterized for

the type of insertion. For that purpose the Southern blot of EcoRI digested genomic DNA isolated from these clones has been performed. Although one of these clones gave signal of distinct size other than expected from unmodified *IL-2* loci, the final clonclusion was that only random DNA incorporations took place. Thus, the original plasmid is now being modified by addition of TK cassette at the 3' end, which will allow us to use the positive-negative cell selection technique (PNS). Although none of the resistant clones demonstrated an incorporation of GFP transgene *in locus* sevral clones have shown proper phenotypic characteristics and were added to the reporter cell line collection.

Consequently, it is still desirable to deliver cell line or cell lines genetically engineered to replace one or two alleli of cytokine gene with coding region of GFP reporter gene.

Example 7. Development of the experimental protocol for testing the response of reporter cell lines to xenobiotics.

To verify if responses of reporter cell lines to the set of characterized immunotoxins are detectable and reproducible. To select cell lines for the assemble of the prototypic panel of reporter cell lines. To prepare standardized experimental protocol that could be used for testing substance of interest employing this prototypic panel of reporter cell lines.

Methodology and study materials

The cell culture and assay condition will be optimized to obtain comparable level of baseline fluorescence and the best signal to noise ratio. The preferable format for these experiments will be testing cells placed in multiwell plate. Selected immunomodulatory substances will be added at increasing concentrations and the expression of GFP in tested cells will be measured. For that purpose activated or resting cells will be incubated in the presence or absence of tested compounds and the kinetic assay of GFP specific fluorescence will be performed. This fluorescence signal will provide information on possible modulation of cellular response leading to enhancement or inhibition of cytokine expression. Based on obtained data a common experimental protocol will be designed which would allow the usage of several cell lines representing different cell lineage in a single assay. Results will facilitate the selection of cell lines for assembling and testing of the entire panel of reporter cell lines, the prototype of a cell chip. The cell line selected for the cell chip has to fulfill following criteria:

The phenotypic characteristics identical with the original cell line

The ability to generate detectable fluorescence signal in response to lineage specific immunological and pharmacological stimuli

The low level of spontaneous GFP associated fluorescence

The stability of all desired characteristics observed following several passages (several months of culture)

This is desirable to obtain the knowledge critical for optimization of the prototype cell chip. Specifically generation of experimental data on the level of sensitivity of fluorescence detection of changes in gene expression observed with selected xenobiotics.

The resultant data shall point to the possible problems and suggest future development. Based on the result obtained with commercially available fluoroimagers we may also specify the technical requirements of fluorescence detector that may further improve this assay.

Propagation of reporter cell lines

Cell clones were propagated and transferred for banking. More than fifty reporter GFP transfected cell clones with the proper phenotypic characteristics are stored in multiple aliquots in a cell banking facility.

Phenotypic characterisation of reporter cell lines

Since TPA/ionomycin treatment only mimics T-cell activation to a limited extent, other modes of activation of EL4 derived reporter cell lines were tested. The feasibility of anti-CD3 antibody for activation of EL4 T-cells was tested. In series of experiments EL4 reporter clones were cultured on plates coated with anti-CD3 antibodies. FACS analysis of GFP expression showed that the anti-CD3 treatment did not induce GFP expression. To test the functionality of the anti-CD3 plate a proliferation assay was then performed. The results show that treatment with the anti-CD3 antibody inhibited proliferation of the EL4 cells, as has been demonstrated earlier. The usage of Concavalin A, another T cell activator is currently being tested.

In order to correlate the GFP expression data with effects on the expression of endogenous cytokine mRNA, RT-PCR analysis was performed. Non-transfected EL4 and BW5147.3 cells were activated with TPA/ionomycin or anti-CD3 and RNA was prepared. Following cDNA synthesis PCR was performed to detect expression of IL-2, IFNγ, and GAPDH (control). The

results generally agree with the GFP expression data. In BW5147.3 cells neither IL-2, nor IFNγ mRNA, could be detected in non-induced cells or after activation. In EL4 cells, IL-2 and IFNγ expression could be induced by TPA/ionomycin, but not anti-CD3.

Detection of green fluorescent protein

Two laboratories have compared different methods to measure GFP fluorescence in reporter cell lines. Preliminary testing using the cells with constitutive expression of GFP, have showed that detection of GFP with fluorescence ELISA readers is possible. At NIPH, FluoStar and FACS were compared, while at RIVM FluoStar, FluoImager and fluorescence microscopy were compared. Good quantitative correlations were seen between the results obtained using FluoStar and FACS, and between FluoStar and FluoImager, respectively, and a good qualitative correlation to fluorescence microscopy.

Cells - viability testing

The work to determine the toxic range of all the chemicals listed in Table II for all the cell lines developed in the project has progressed. The basic cytotoxicty assay employed was LDH release. Several cytotoxicity assays were performed independently in two laboratories. Data, which are presented in Fig. 20 and Fig. 21, respectively, were compared and analyzed and will allow us to decide on the range of concentrations to be used for testing of the Fluorescence Cell Chip. In addition, the cytotoxicity associated with the solvent was also tested to eliminate the possibility of interference in the test outcome.

Preliminary tests of reporter cell lines for their responses to model xenobiotics.

The effect of **tetrachloroplatinate** and **nickel sulfate** on reporter cell line J/p4-5'IL1β-dEGFP/4 was investigated. First, the NO levels in the supernatants of tested cells exposed to these substances were measured. Both, tetrachloroplatinate and nickel sulfate induced elevated levels of NO. Although reporter cells stimulated with these compounds didn't show noticeable fluorescence under microscope, upregulation of mRNA for GFP in the clone stimulated for 6h with tetrachloroplatinate (100μM) was observed (Fig. 22).

The effect of **Cyclosporin A**, **Rapamycin** and **TCDD** (dioxin) on selected lymphocytic reporter cell lines were determined independently in three laboratories. EL4 and reporter cells for IL-2, EL/p1-5'IL2-EGFP/3 and EL/p1-5'IL2-dEGFP/6, were treated with Cyclosporin A,

Rapamycin and TCDD (dioxin) to test the effect on basal and induced GFP expression. For EL4 derived cells basal GFP expression was not affected following treatment with any of the above chemicals. Activation of EL4 cells with TPA/ionophore resulted in the upregulation of EGFP fluorescence as observed by FACS, fluorescence microscope and Fluorostar plate reader. Cyclosporin A was shown to completely inhibit the activation induced GFP expression down to basal levels (Fig. 23, Fig. 24). This inhibitory effect of Cyclosporine was observed independently in all three laboratories performing such experiments. Furthermore, this inhibition of EGFP expression was detected with all three techniques emplyed for EGFP measurements (i.e. FACS, fluorescence microscope, and Fluorostar). Rapamycin or TCDD did not have any effect on the activation induced GFP expression. Thus the important proof of the concept of Fluorescence Cell Chip testing has been obtained, namely the microplate based readout detected the presence of model immunosupresive xenobiotic (Cyclosporin A; Fig. 24). Additional RT-PCR based analysis showed that Cyclosporin A treatment inhibited the upregulation of expression of IL-2 and IFNγ in activated reporter cell lines, while Rapamycin or TCDD had no effect (Fig. 25). These results show that Cyclosporin A inhibited in parallel the induction of endogenous cytokine genes and the GFP reporter transgene.

BW5147.3 derived GFP transfected cell clones have been also used to test the effects of immunomodulatory compounds. Cyclosporin A, Rapamycin and TCDD did not affect the basal GFP expression and since the GFP expression could not be induced, the effect on activated expression could not be analysed.

Example 8. Pre-validation of the new test against available data on animal and human immunotoxicity. Prototype of cell-chip.

Methodology and study materials

Work will start from providing all participants with the panel of reporter cell lines-the prototype of a cell chip assembled in previos examples. The standardized experimental protocol developed in Example 7 for performing such test will be implemented in all collaborating laboratories. All laboratories will next employ the same set of tested substances. In this example it will be used not only the set of xenobiotics with defined immunotoxic properties (see Table 2) but also a set of inert substances that are unlikely to have any

immunotoxic effects *in vivo*. All the experiments will be performed with the entire set of reporter cell lines, the prototype cell chip. Collection of several patterns of response for these xenobiotic will allow to compare these patterns with available data. Comparison of patterns generated by model immunotoxins, substances that may be classified as irritants but do not posses immunomodulatory activity and control inert compounds will be performed. This analysis shall reveal if the new technology is capable to distinguish immunotoxins from other xenobiotics. Testing known and unknown (blind) samples of xenobiotic in parallel experiments will next be performed in all laboratories. A comparison of data obtained independently in participating laboratories will provide preliminary data on reproducibility of responses of reporter cell lines, and the sensitivity of this technology to minor differences in experimental protocols. It is desirable to obtain information in a format of "two dimensional" pattern that describes the action of several "model xenobiotics" (substances already known for their immunomodulatory activities in vivo) on different genes in various cell lineages.

Results

Preparation for the experimental work

Fluorstar Galaxy Multiwell (BMG Labtechnologies) fluorescence, luminescence, and an absorbance reader were used for fluorescence detection. Two laboratories received two cell lines 3T3-L1/CMV-EGFP and HEL-30/CMV-EGFP and employed them for testing both the cell culture and the fluorescence assay protocols.

Exposure to xenobiotics / The Cell Chip lay-out

Cells were plated in 24 well microtiter plates at a density of $0.5x10^6$ cells/mL and with a final volume of 1.5 mL/well. Two controls were included for each cell line, in each experiment. One control consisted of cells in growth medium and the second control was cells in the presence of the induction mix (Ionomycin calcium salt, (cat.no#1-0634, Sigma) and 10 ng/mL PMA *(Sigma)*. In some cases, induction mix contained only ionomycin or LPS at concentration 100 ng/ml rather than PMA and ionomycin. Cells were incubated with different chemicals at the concentration that led to 10% cytotoxicity and concentrations 10x and 100x more diluted in the absence and presence of the induction mix. Cells were incubated in a humidified atmosphere, 37°C and 5% CO_2, and after 4h 0.5 mL was removed from each test

sample and analyzed by flow cytometry. The remaining sample was further incubated for 20h (total 24h) before flow cytometry.

Flow cytometry

Samples were analyzed in an EPICS® XL-MCL Coulter flow cytometer with Expo v.2 Analysis Software/ Expo32 Analysis Software (Applied Cytometry Systems, Sheffield, UK). or any other suitable flow cytometer. Viable and dead cells were gated separately and results were determined based on fluorescence associated with viable cells only. Regions were set in control cells and the same regions were used in induction controls and chemically challenged cells. Percent positive cells and fluorescence intensity were noted and used in further calculations.

Data presentation

Index numbers were calculated from the median fluorescence intensity of viable cells. Cells incubated in the absence or presence of ionomycin/PMA were analysed separately. Each clone incubated with or without ionomycin/PMA, but without further chemicals/compounds was defined as index number = 1, and the index numbers for the other exposures were calculated from this. Experiments with the same exposures were pooled to perform statistical analysis, using SigmaStat 2.03 statistical analysis software (SPSS Inc., Chicago, Illinois, USA).

Statistical analysis

One way ANOVAs (one way analysis of variance) were performed. In cases where normality failed, we used Kruskal-Wallis One Way Analysis of Variance on Ranks. If the differences between groups were statistically significant ($p<0.05$), we continued with tests for multiple comparisons. In normally distributed data we used Bonferroni t-test and multiple comparisons versus the control. The control was chosen to be the sample incubated in the presence or absence of ionomycin/PMA only. When normality failed we used Dunnett's Method for multiple comparisons versus the control.

Chemicals used for testing the prototype cell chip

Cyclosporin A (for molecular biology, Tolypocladium inflatum, minimum purity 95%, Sigma) was diluted in ethanol to a stock concentration of 5 mg/mL and kept at –20°C. The

stock solution was diluted in medium prior to experiments. Rapamycin (minimum purity 95%, Sigma) was dissolved in DMSO to a concentration of 2 mg/mL and kept at –20°C. The stock solution was diluted in medium prior to experiments. Pentamidine isethionate salt (Sigma) was dissolved in DMSO to a final concentration of 25 mg/mL and kept at –20°C. The stock solution was diluted in medium prior to experiments. (+/-)- Thalidomide (purity >98%, Sigma). A 0.2 M stock solution of Thalidomide in DMSO was prepared and kept at -20°C. Prior to experiments, the stock solution was diluted in medium, which led to a white precipitate. The solution was resuspended and used for further dilutions. Bis (tri-n-butyltin) oxide (TBTO) (purity 96%, Aldrich) was diluted in ethanol to a stock concentration of 10 mM, kept at -20°C and diluted in medium before use. House dust mite D- pteronyssinus (Alutard SQ Depot allergen extract) (suspension for injection, 100 000 SQ-U/mL solution, ALK-Abelló, Hørsholm, Denmark) was kept at 4-8°C as instructed by the manufacturer and diluted in medium directly before use. 1-Chloro-2,4-dinitrobenzene (DNCB) (purity minimum 98%, Sigma) was diluted in ethanol to a stock solution of 2 mM, kept at –20°C and diluted in medium before use. Benzocaine (Ethyl-4-Aminobenzoate, Sigma) was diluted to a 0.5 M stock solution in ethanol, kept at –20°C and diluted in medium before use. Tolylene 2,4-diisocyanate (TDI) (Sigma, purity 95%). On the day of the experiment, TDI was first dissolved in DMSO to a concentration of 0.1 M. This stock solution was further diluted in medium to 1 mM, before further dilution in medium. It is noteworthy that the TDI / DMSO solution is of higher density than medium and sinks to the bottom of the tube as well as becoming insoluble. The white precipitate was resuspended to an even suspension before dilution. Potassium tetrachloro-platinate (II) (purity 99.99%, Aldrich) was diluted in medium to a stock solution of 30 mM, kept at –20°C and diluted in medium before use. Penicillin G (Benzylpenicillin) sodium salt (activity >1477 U/mg, Sigma) was diluted in medium to a stock solution of 200 mM, kept at –20°C and further diluted in medium before use. SDS (purity >85%, Merck) was diluted in DMSO to a stock solution of 100 mM, kept at –20°C and diluted in medium before use. Mercury (II) chloride (minimum purity 99.5%, Merck) was diluted in ethanol to a stock solution of 6 mM, kept at –20°C and diluted in medium before use.

Prototype of cell-chip

The following cell lines: EL/pCA-dEGFP/9, EL/p1-5'IL2-dEGFP/6, EL/p2-5'IL4-dEGFP/2, EL/p1-5'IFNγ-dEGFP/3, and EL/p2-5'IL10-dEGFP/5, obtained by transfection with the DNA constructs containing promoter region derived from β-actin, IL2, IL4, INF-γ, and IL10 were employed to design the prototype cell chip. The prototype cell chip was used to test the activity of different substances.

Results of testing the effect of different substances using the prototype cell chip

Immunosuppressants

Cyclosporin A (CsA) and Rapamycin are well-known immunosuppressive drugs used in organ transplantation. Pentamidine is used as an antiprotozoal drug, but has also been shown to reduce expression of several cytokines. Thalidomide is used as an anti-inflammatory drug. Bis(tri-n-butyltin)oxide (TBTO) has also been shown previously to be immunosuppressive, both in vivo and in vitro.

Cyclosporin A

Figure 26 presents results of testing the effect of Cyclosporin A using the prototype cell chip: cells transfected with the regulatory elements from actin (black bars), IL-2 (grey bars), IL-4 (wide striped bars), IL-10 (diamond bars) and IFN-γ (narrow striped bars) fused to EGFP were exposed to 1 μM CsA, which led to 10% cytotoxicity, and 1:10 or 1:100 dilution of this for 24 h in the absence (A) or presence (B) of ionomycin/PMA (1 μM / 10 ng/mL). In non-induced cells (A) at the highest concentration, we found statistically significant differences compared to the control for actin, IL-4 and IL-10. At the 1:10 dilution, only IL-4 and IL-10 were statistically significantly different from their control. In stimulated cells (B) expression of all genes tested were statistically significantly different from their control, except for IL-4 at the 1:100 dilution. The graphs show the mean values with SEM (n=3). Statistically significant findings with $p<0.05$ are noted by *, $p<0.01$ are noted by ** and $p< 0.001$ are noted by ***. The dashed line represents the control level.

In the absence of ionomycin/PMA, we found a statistically significant dose-dependent decrease in IL-4 and IL-10 at 1 and 0.1 μM. Surprisingly, we found a significantly higher

expression of actin at the highest concentration (1 μM) (fig 26A). In the presence of ionomycin/PMA we found significantly lower induction in all clones, at all concentrations, except for IL-4 at the lowest concentration (fig 26B). The suppressive action of CsA in stimulated cells was confirmed for IL-2 and IFN-γ, by using clones transfected with the same regulatory elements, but fused to a stabile form of EGFP. In these clones in the presence of ionomycin/PMA, CsA exposure resulted in a dose-dependent reduction of IL-2 expression at 0.01 μM and 0.1 μM ($p < 0.01$) and of IFN-γ expression at 0.01 μM ($p < 0.01$) and 0.1 μM ($p < 0.001$). In the absence of ionomycin/PMA these cells failed to show exposure effects (data not shown).

Rapamycin

Figure 27 presents results of testing the effect of rapamycin using the prototype cell chip: cells transfected with the regulatory elements from actin (black bars), IL-2 (grey bars), IL-4 (wide striped bars), IL-10 (diamond bars) and IFN-γ (narrow striped bars) fused to EGFP were exposed to 10 μg/mL rapamycin, which led to 10% cytotoxicity, and 1:10 or 1:100 dilution of this for 24 h in the absence (A) or presence (B) of ionomycin/PMA (1 μM / 10 ng/mL). In non-induced cells (A) at the highest concentration, we found a statistically significant decrease in fluorescence compared to the control for IL-4 and IL-10. At the 1:10 dilution, IL-4 was still inhibited while actin was statistically significantly increased. In stimulated cells (B) at the highest concentration IL-2, IL-4 and IL-10 were statistically significantly different from their controls. At the 1:10 and 1:100 dilutions, actin and IL-2 showed a statistically significant increase compared to their controls. The graphs show the mean values with SEM (n=3 for IL-10 and IFN-γ, n=4 for actin and IL-4, n=5 for IL-2). Statistically significant findings with $p<0.05$ are noted by *, $p<0.01$ are noted by ** and $p< 0.001$ are noted by ***. The dashed line represents the control level.

In the absence of ionomycin/PMA, we found a significant reduction of IL-4 at all tested concentrations of rapamycin (10, 1 and 0.1 μg/mL), while IL-10 showed expression only at the highest concentration. However, we also found a significant increase of actin at the two lowest concentrations (fig 27A). In the presence of ionomycin/PMA, at the highest

concentration, rapamycin induced suppression of IL-2, IL-4 and IL-10. IFN-γ was also suppressed at the highest concentration, but statistical analysis did not confirm the trend, due to low power of the test (fig 27B). At the two lowest concentrations (1 and 0.1 μg/ml) IL-2 and actin showed a significant increase. IL-10 and IFN-γ showed a trend towards an increase, while IL-4 was not affected.

Pentamidine

Figure 28 presents results of testing the effect of rapamycin using the prototype cell chip: cells transfected with the regulatory elements from actin (black bars), IL-2 (grey bars), IL-4 (wide striped bars), IL-10 (diamond bars) and IFN-γ (narrow striped bars) fused to EGFP were exposed to 30 μg/mL pentamidine, which led to 10% cytotoxicity, and 1:10 or 1:100 dilutions of this for 24 h in the absence (A) or presence (B) of ionomycin/PMA (1 μM / 10 ng/mL). In non-induced cells (A) at the highest concentration and 1:10 dilution, we found statistically significant differences compared to the control for actin and IL-10. In stimulated cells (B) at the highest concentration all tested cytokines were statistically significantly different from their controls. At the 1:10 dilution, IL-2, IL-4 and IL-10 were statistically significantly different from their controls. The graphs show the mean values with SEM (n=3 except n=4 for IL-2). Statistically significant findings with $p<0.05$ are noted by *, $p<0.01$ are noted by ** and $p< 0.001$ are noted by ***. The dashed line represents the control level.

We found an increase in actin at the two highest concentrations of pentamidine (30 and 3 μg/mL), while IL-10 was clearly inhibited at these concentrations in the absence of ionomycin/PMA (fig. 28A). In the presence of ionomycin/PMA, pentamidine exerted suppressive activity, since at the highest concentration all cytokines were suppressed, while at the 1:10 dilution all cytokines except IFN-γ were suppressed (fig. 28B). The expression of actin was not altered in the presence of pentamidine at any of the concentrations tested in ionomycin/PMA activated cells.

Thalidomide

We did not find thalidomide to be toxic in the LDH-assay and used 1 mM as the highest concentration. Cells exposed to thalidomide in the absence of ionomycin/PMA did not show

any significant changes compared to their controls, except for actin at 1 mM ($p<0.05$) and even there the reduction was very small (5-10 % inhibition; data not shown). The same result was apparent in the presence of ionomycin/PMA, with only actin inhibited ($p<0.01$). However, experiments using IL-2 and IFN-γ fused to a stabile form of EGFP in the absence of ionomycin/PMA showed a significant inhibition of 10% for IL-2 at 1 mM ($p<0.05$). In the presence of ionomycin/PMA both IL-2 and IFN-γ showed a dose-dependent inhibition at 1 mM ($p<0.001$). Thalidomide exposure did not show detectable effects at the two lowest concentrations.

Bis(tri-n-butyltin)oxide (TBTO)

Experiments using IL-2 and IFN-γ fused to a stabile form of EGFP, in the absence of ionomycin/PMA at any TBTO concentration tested (50, 5 and 0.5 μM), failed to show altered expression of both IL-2 and IFN-γ. In the presence of ionomycin/PMA, a statistically significant inhibition of IL-2 at the 1:10 dilution 10% (5 nM) was found ($p<0.01$).

Allergens and autoimmunity inducing agents

We also wanted to examine the possible use of the "Cell Chip" panel to detect (and distinguish) different kinds of allergens. Substances representing three classes of allergens were included: (1) IgE-mediated respiratory allergy to protein allergens, exemplified by the mite allergen Der p I, (2) contact allergy to low molecular weight substances mediated by hapten-protein conjugate specific T lymphocytes, exemplified by 1-chloro-2,4-dinitrobenzene (strong allergen) and benzocaine (weak allergen), and (3) low-molecular weight chemical allergens, causing clinical symptoms similar to IgE-mediated allergy but with unknown mechanisms, exemplified by tolylene 2,4-diisocyanate where specific IgE is demonstrable only in a minority of cases and potassium tetrachloroplatinate where specific IgE is regularly demonstrable. Penicillin G was included because it is known to induce drug hypersensitivity in humans. Furthermore, an irritant (sodium dodecyl sulphate) was included, because the distinction between irritants and contact allergens is an important problem in contact allergy testing. Finally, mercuric chloride was included since it is known to induce Th1 and Th2 subsets leading to autoimmunity.

Der p- dust mite allergen

Figure 29 presents results of testing the effect of Der p-mite allergen using the prototype cell chip: cells transfected with the regulatory elements from actin (black bars), IL-2 (grey bars), IL-4 (wide striped bars), IL-10 (diamond bars) and IFN-γ (narrow striped bars) fused to EGFP were exposed to 3000 SQU/mL Der p, as the highest concentration, and 1:10 or 1:100 dilution of this for 24 h in the absence (A) or presence (B) of ionomycin/PMA (1 μM / 10 ng/mL). In non-induced cells (A) at the highest concentration, a statistically significant increase in fluorescence compared to the control was found for all clones except IL-10. In the presence of stimulation no alterations in fluorescence were found (B). The graphs show the mean values with SEM (n=3 for IL-10, n=4 for actin and IL-4, n=5 for IFN-γ and n=7 for IL-2). Statistically significant findings with $p<0.05$ are noted by *. The dashed line represents the control level.

We used a Der p solution produced for skin prick testing, and added it to cells at 3000, 300 and 30 Standard Quality U/mL. We found that Der p increased the fluorescence at the highest concentration in the absence of ionomycin/PMA for all clones except IL-10 (fig 29A). No significant changes were observed in the presence of ionomycin/PMA (fig 29B).

1-Chloro-2,4-dinitrobenzene (DNCB)

Figure 30 presents results of testing the effect of DNCB using the prototype cell chip: cells transfected with the regulatory elements from actin (black bars), IL-2 (grey bars), IL-4 (wide striped bars), IL-10 (diamond bars) and IFN-γ (narrow striped bars) fused to EGFP were exposed to10 μM DNCB, which led to 10% cytotoxicity, and 1:10 or 1:100 dilutions of this for 24 h in the absence (A) or presence (B) of ionomycin/PMA (1 μM / 10 ng/mL). In non-induced cells (A) at the highest concentration, we found a statistically significant increase in fluorescence compared to the control for actin, IL-2, IL-4 and IFN-γ. In the presence of stimulation, no statistically significant effects were found (B). The graphs show the mean values with SEM (n=3). Statistically significant findings with $p<0.05$ are noted by *, $p<0.01$ are noted by ** and $p< 0.001$ are noted by ***. The dashed line represents the control level.

In the absence of ionomycin/PMA, at the highest concentration (10 μM) of DNCB, an increased expression was seen for all clones, except IL-10. The largest increase was observed in cells transfected with actin regulatory elements (fig 30A). In the presence of ionomycin/PMA, no statistically significant changes were found but the trend was towards a decrease in fluorescence at the highest concentration (fig 30B). For cells transfected with a stable form of EGFP, similar results were found. DNCB failed to alter expression of IL-2 or IFN-γ in the presence of ionomycin/PMA, while in the absence of these stimuli IFN-γ showed an apparently dose-dependent increase reaching statistical significance at the highest concentration ($p<0.001$).

Benzocaine

Figure 31 presents results of testing the effect of benzocaine using the prototype cell chip: cells transfected with the regulatory elements from actin (black bars), IL-2 (grey bars), IL-4 (wide striped bars), IL-10 (diamond bars) and IFN-γ (narrow striped bars) fused to EGFP were exposed to 1 mM benzocaine as the highest concentration and 1:10 or 1:100 dilution of this for 24 h in the absence (A) or presence (B) of ionomycin/PMA (1 μM / 10 ng/mL). In non-induced cells (A) at the highest concentration, a statistical increase in fluorescence compared to the control for actin is apparent. In the presence of stimulation, IL-2, IL-10 and IFN-γ are reduced at the highest concentration. IL-2 and IL-10 are also reduced at the 1:10 dilution (B). The graphs show the mean values with SEM (n=3). Statistically significant findings with $p<0.05$ are noted by *, $p<0.01$ are noted by ** and $p< 0.001$ are noted by ***. The dashed line represents the control level.

We did not find benzocaine to be toxic in the LDH-assay and used 1 mM as the highest concentration. In the absence of ionomycin/PMA, only actin at the highest concentration was significantly increased (fig 31A). In the presence of ionomycin/PMA, a statistically significant inhibitory effect on IL-2 and IL-10 was seen at the two highest concentrations. IFN-γ was also inhibited, but only at the highest concentration (fig 31B).

3.2.4 Tolylene 2,4-diisocyanate (TDI)

Figure 32 presents results of testing the effect of TDI using the prototype cell chip: cells transfected with the regulatory elements from actin (black bars), IL-2 (grey bars), IL-4 (wide striped bars), IL-10 (diamond bars) and IFN-γ (narrow striped bars) fused to EGFP were exposed to 500 μM TDI, which led to 10% cytotoxicity, and 1:10 or 1:100 dilutions of this for 24 h in the absence (A) or presence (B) of ionomycin/PMA (1 μM / 10 ng/mL). In non-induced cells (A) at the highest concentration, we found a statistically significant increase in fluorescence compared to the control for IFN-γ. However, in stimulated cells (B) at the highest concentration IL-4 was the only one that was statistically significantly different from the control. The graphs show the mean values with SEM (n=3 for actin, IL-4 and IL-10, n=7 for IL-2 and IFN-γ). Statistically significant findings with $p<0.05$ are noted by *. The dashed line represents the control level.

Cells were exposed to TDI at 500, 50 or 5 μM. In the absence of ionomycin/PMA, only IFN-γ showed a significant induction and only at the highest concentration (fig 32A). However, in the presence of ionomycin/PMA a statistically significant increase was seen for IL-4 and again only at the highest concentration (fig 32B).

Potassium tetrachloroplatinate - K_2PtCl_4

In experiments using IL-2 or IFN-γ fused to a stabile form of EGFP, cells were exposed to K_2PtCl_4 at 100, 10 or 1 μM. In the presence of ionomycin/PMA, exposure resulted in a dose-dependent reduction of IL-2 and IFN-γ expression. Statistically significant values were found at 100 μM, with $p< 0.001$ for IL-2 and $p< 0.05$ for IFN-γ. In the absence of ionomycin/PMA, exposure effects were not observed.

Penicillin G

We did not find penicillin G to be toxic. At the tested concentrations (10, 1 and 0.1 mM), penicillin G exposure failed to alter expression of either IL-2 or IFN-γ in cells transfected with IL-2 or IFN-γ fused to a stabile form of EGFP. This was found both in the presence and absence of ionomycin/PMA.

Sodium dodecyl sulphate (SDS)

Cells were exposed to SDS at 300, 30 or 3 μM. In cells transfected with IL-2 or IFN-γ fused to a stabile form of EGFP, in the presence of ionomycin/PMA, exposure resulted in an apparently dose-dependent reduction of IL-2 expression, with $p<0.05$ at the highest concentration. IFN-γ expression was, however, not affected. In the absence of ionomycin/PMA exposure effects were not observed.

Mercuric chloride - $HgCl_2$

Cells transfected with IL-2 or IFN-γ fused to a stabile form of EGFP were exposed to mercuric chloride at 6, 0.6 and 0.06 μM. In the presence of ionomycin/PMA exposure resulted in an apparently dose-dependent reduction of IFN-γ expression reaching statistical significance at 6 μM ($p< 0.05$). IL-2 expression was, however, not affected. In the absence of ionomycin/PMA exposure effects were not observed.

Conclusion

Described embodiment of in vitro immunotoxicity screening system has several important advantages. Other methods to measure cytokine expression, such as RT-PCR or ELISA are more time-consuming. Exemplified system is based on fluorescence technology, which enables concurrent viability assessment. Several methods for detection of fluorescence are available, such as Fluostar plate reader, fluorescence microscopy and flow cytometry. Although the project started off employing both flow cytometry and plate-based assays, we soon decided to use flow cytometry based on the advantages of concurrent assessment of viability, the possibility to measure fluorescence intensity per cell and possible effects on cell shape and size.

Several quality controls have been performed and are described elsewhere. Firstly, the transfected cell lines and the parent cell line have similar gene expression, both basal and in response to stimulation. Secondly, the fluorescence intensity is correlated to EGFP gene expression and parent gene expression (parent meaning e.g. the IL-4 gene in IL-4/EGFP transfected) and finally the cytokine levels correlate to the fluorescence intensities.

The tested panel consisted of only one type of cells (EL4; T-cells). An increased selection of cell types and cytokines will most probably enhance the precision and sensitivity of the "Cell Chip". One limitation with exemplified in vitro systems is the absence of antigen presenting cells. For a substance to give an effect in exemplified system, it must interact directly with the cells. A high level of protein in the medium might possibly reduce substance-cell interactions. Immunosuppressive compounds often exert their effects directly on T-cells, suggesting that the T-cell lymphoma used in the exemplified embodiment is a suitable target cell line to evaluate immunosuppressive potential. Sensitizers, however, often exert their effects on other cell types such as keratinocytes (KC) and dendritic cells (DC), suggesting that T-cells may not be a suitable target to assess sensitising potential. This notion is supported by the fact that the exemplified embodiment that employs a T cell line was more successful in identifying immunosuppressive compounds than sensitising compounds. Cell lines of KC and DC origin should thus be included in the panel of cells used in the cell chip approach.

Most in vitro exposure models lack organ architecture, thereby diminishing the possibilities for cell-cell interaction, especially if one of the cell types is sessile. This lack of interaction often hampers cell maturation, precluding the evaluation of the sensitivity of cells to toxic compounds at different stages of development.

Both stable and destabilised EGFP have provided meaningful results. A choice between these types requires additional testing. In conclusion, exemplified embodiment of "cell chip" approach may be useful as a pre-screen to identify immunotoxicity. Cell lines derived from other origins and additional compounds, shall be tested and possibly used in other embodiments of the "cell chip" according to the invention.